

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : <b>C12N 15/62, 1/19, 1/20, 11/16 // 1:19, C12R 1:465</b>		A1	(11) International Publication Number: <b>WO 94/18330</b> (43) International Publication Date: <b>18 August 1994 (18.08.94)</b>
(21) International Application Number: <b>PCT/EP94/00427</b> (22) International Filing Date: <b>10 February 1994 (10.02.94)</b>		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(30) Priority Data: <b>93200350.2 10 February 1993 (10.02.93) NL</b>		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for AU BB CA GB IE LK MN MW NZ SD only): <b>UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4 4BQ (GB).</b> (71) Applicant (for all designated States except AU BB CA GB IE LK MN MW NZ SD US): <b>UNILEVER N.V. [NL/NL]; Veena 455, NL-3013 AL Rotterdam (NL).</b>			
(72) Inventors; and (75) Inventors/Applicants (for US only): <b>FRENKEN, Leon, Gerardus, J. [NL/NL]; Geldersestraat 90, NL-3011 MP Rotterdam (NL). DE GEUS, Pieter [NL/NL]; Bocier 24, NL-2991 KB Barneveld (NL). KLIS, Franciscus, Maria [NL/NL]; Bendenlangs 102, NL-1025 KL Amsterdam (NL). TOSCHKA, Holger, York [DE/DE]; Langnese-Iglo, BR3, Aekern 1, D-48734 Reken (DE). VERRIPS, Cornelis, Theodorus [NL/NL]; Hagedoorn 18, NL-3142 KB Maassluis (NL).</b>			

(54) Title: **IMMOBILIZED PROTEINS WITH SPECIFIC BINDING CAPACITIES AND THEIR USE IN PROCESSES AND PRODUCTS**

(57) Abstract

A method is provided for immobilizing a binding protein capable of binding to a specific compound, using recombinant DNA techniques for producing said binding protein or a functional part thereof. The binding protein is immobilized by producing it as part of a chimeric protein also comprising an anchoring part derivable from the C-terminal part of an anchoring protein, thereby ensuring that the binding protein is localized in or at the exterior of the cell wall of the host cell. Suitable anchoring proteins are yeast  $\alpha$ -agglutinin, FLO1 (a protein associated with the flocculation phenotype in *S. cerevisiae*), the Major Cell Wall Protein of lower eukaryotes, and a proteinase of lactic acid bacteria. For secretion the chimeric protein can comprise a signal peptide including those of  $\alpha$ -mating factor of yeast  $\alpha$ -agglutinin of yeast, invertase of *Saccharomyces*, inulinase of *Kluyveromyces*,  $\alpha$ -amylase of *Bacillus*, and proteinase of lactic acid bacteria. Also provided are recombinant polynucleotides encoding such chimeric protein, vectors comprising such polynucleotide, transformed microorganisms having such chimeric protein immobilized on their cell wall, and a process for carrying out an isolation process by using such transformed host, wherein a medium containing said specific compound is contacted with such host cell to form a complex, separating said complex from the medium and, optionally, releasing said specific compound from said binding protein.

BEST AVAILABLE COPY

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

Title: **Immobilized proteins with specific binding capacities and their use in processes and products**

**Background of the invention**

5 The pharmaceutical, the fine chemicals and the food industry need a number of compounds that have to be isolated from complex mixtures such as extracts of animal or plant tissue, or fermentation broth. Often these isolation processes determine the price of the product.

Conventional isolation processes are not very specific and during the isolation 10 processes the compound to be isolated is diluted considerably with the consequence that expensive steps for removing water or other solvents have to be applied.

For the isolation of some specific compounds affinity techniques are used. The advantage of these techniques is that the compounds bind very specifically to a 15 certain ligand. However these ligands are quite often very expensive.

To avoid spillage of these expensive ligands they can be linked to an insoluble support. However, often linking the ligand is also expensive and, moreover, the functionality of the ligand is often affected negatively by such procedure.

So a need exists for developing cheap processes for preparing highly effective 20 immobilized ligands.

**Summary of the invention**

The invention provides a method for immobilizing a binding protein capable of binding to a specific compound, comprising the use of recombinant DNA techniques 25 for producing said binding protein or a functional part thereof still having said specific binding capability, said protein or said part thereof being linked to the outside of a host cell, whereby said binding protein or said part thereof is localized in the cell wall or at the exterior of the cell wall by allowing the host cell to produce and secrete a chimeric protein in which said binding protein or said functional part 30 thereof is bound with its C-terminus to the N-terminus of an anchoring part of an anchoring protein capable of anchoring in the cell wall of the host cell, which anchoring part is derivable from the C-terminal part of said anchoring protein.

Preferably, the host is selected from Gram-positive bacteria and fungi, which have a cell wall at the outside of the host cell, in contrast to Gram-negative bacteria and cells of higher eukaryotes such as animal cells and plant cells, which have a membrane at the outside of their cells. Suitable Gram-positive bacteria comprise

5 lactic acid bacteria and bacteria belonging to the genera *Bacillus* and *Streptomyces*. Suitable fungi comprise yeasts belonging to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia* and *Saccharomyces*, and moulds belonging to the genera *Aspergillus*, *Penicillium* and *Rhizopus*. In this specification the group of fungi comprises the group of yeasts and the group of moulds, which are also known as

10 lower eukaryotes. In contrast to the cells in plants and animals, the group of bacteria and lower eukaryotes are also indicated in this specification as microorganisms.

The invention also provides a recombinant polynucleotide capable of being used in a method as described above, such polynucleotide comprising (i) a structural gene encoding a binding protein or a functional part thereof still having the specific

15 binding capability, and (ii) at least part of a gene encoding an anchoring protein capable of anchoring in the cell wall of a Gram-positive bacterium or a fungus, said part of a gene encoding at least the anchoring part of said anchoring protein, which anchoring part is derivable from the C-terminal part of said anchoring protein.

The anchoring protein can be selected from  $\alpha$ -agglutinin,  $\alpha$ -agglutinin, *FLO1*, the

20 Major Cell Wall Protein of a lower eukaryote, and proteinase of lactic acid bacteria. Preferably, such polynucleotide further comprises a nucleotide sequence encoding a signal peptide ensuring secretion of the expression product of the polynucleotide, which signal peptide can be derived from a protein selected from the  $\alpha$ -mating factor of yeast,  $\alpha$ -agglutinin of yeast, invertase of *Saccharomyces*, inulinase of

25 *Kluyveromyces*,  $\alpha$ -amylase of *Bacillus*, and proteinase of lactic acid bacteria. The polynucleotide can be operably linked to a promoter, which is preferably an inducible promoter.

The invention further provides a recombinant vector comprising a polynucleotide according to the invention, a chimeric protein encoded by a polynucleotide

30 according to the invention, and a host cell having a cell wall at the outside of its cell and containing at least one polynucleotide according to the invention. Preferably at least one polynucleotide is integrated in the chromosome of the host cell. Another

embodiment of this part of the invention is a host cell having a chimeric protein according to the invention immobilized in its cell wall and having the binding protein part of the chimeric protein localized in the cell wall or at the exterior of the cell wall.

- 5 Another embodiment of the invention is a process for carrying out an isolation process by using an immobilized binding protein or functional part thereof still capable of binding to a specific compound, wherein a medium containing said specific compound is contacted with a host cell according to the invention under conditions whereby a complex between said specific compound and said immobilized
- 10 binding protein is formed, separating said complex from the medium originally containing said specific compound and, optionally, releasing said specific compound from said binding protein or functional part thereof.

#### **Brief description of the figures**

- 15 In Figure 1 the composition of pEMBL9-derived plasmid pUR4122 is indicated, the preparation of which is described in Example 1.
- In Figure 2 the composition of plasmid pUR2741 is indicated, which is a derivative of published plasmid pUR2740, see Example 1.
- 20 In Figure 3 the composition of pEMBL9-derived plasmid pUR2968 is indicated. Its preparation is described in Example 1.
- In Figure 4 the preparation of plasmid pUR4174 starting from plasmids pUR2741, pUR2968 and pUR4122 is indicated, as well as the preparation of plasmid pUR4175 starting from plasmids pSY16, pUR2968 and pUR4122. These preparations are described in Example 1.
- 25 In Figure 5 the composition of plasmid pUR2743.4 is indicated. Its preparation is described in Example 2. It contains the 714 bp *PstI-XbaI* fragment given in SEQ ID NO: 12, which fragment encodes an scFv-TRAS fragment of anti-trasolide® antibody 02/01/01.
- 30 In Figure 6 the composition of plasmid pUR4178 is indicated. Its preparation is indicated in Example 2. It contains the above mentioned 714 bp *PstI-XbaI* fragment given in SEQ ID NO: 12. This plasmid is suitable for the expression of a fusion

protein between scFv-TRAS and  $\alpha$ AGG preceded by the invertase signal sequence (SUC2).

In Figure 7 the composition of plasmid pUR4179 is indicated. Its preparation is indicated in Example 2. It contains the above mentioned 714 bp *PstI-XbaI* fragment

5 given in SEQ ID NO: 12. This plasmid is suitable for the expression of a fusion protein between scFv-TRAS and  $\alpha$ AGG preceded by the prepro- $\alpha$ -mating factor signal signal sequence.

In Figure 8 a molecular design picture is given, showing the musk odour molecule traseolide® and a modified musk antigen, described in Example 3.

10 In Figure 9 the composition of plasmid pUR4177 is indicated. Its construction is described in Example 4. Plasmid pUR4177 contains the 734 bp *EagI-XbaI* DNA fragment given in SEQ ID NO: 13 encoding the variable regions of the heavy and light chain fragments from the monoclonal antibody directed against the human chorionic gonadotropin (an scFv-HCG fragment) and is a 2  $\mu$ m-based vector

15 suitable for production of the chimeric scFv HCG- $\alpha$ AGG fusion protein preceded by the invertase signal sequence and under the control of the GAL7 promoter.

In Figure 10 the composition of plasmid pUR4180 is indicated. Its preparation is indicated in Example 4. It contains the above mentioned 734 bp *EagI-XbaI* DNA fragment given in SEQ ID NO: 13 and is a 2  $\mu$ m-based vector suitable for

20 production of the chimeric scFv-HCG- $\alpha$ AGG fusion protein preceded by the prepro- $\alpha$ -mating factor signal sequence and under the control of the GAL7 promoter.

In Figure 11 the composition of plasmid pUR2990, a 2  $\mu$ m-based vector, is indicated, which is suggested in Example 5 as a starting vector for the preparation of plasmid pUR4196 (see Figure 12). Plasmid pUR2990 contains a DNA fragment

25 encoding a chimeric lipase-FLO1 protein that will be anchored in the cell wall of a lower eukaryote and can catalyze lipid hydrolysis.

In Figure 12 the composition of plasmid pUR4196 is indicated. Its preparation is explained in Example 5. It contains a DNA fragment encoding a chimeric protein comprising the scFv-HCG followed by the C-terminal part of the FLO1-protein, and

30 is a vector suitable for the production of a chimeric protein anchored in the cell wall of the host organism and can bind HCG.

In Figure 13 the composition of plasmid pUR2985 is indicated. Its preparation is described in Example 6. It contains a *choB* gene coding for the mature part of the cholesterol oxidase (EC 1.1.3.6) obtained via PCR techniques from the chromosome of *Brevibacterium sterolicum*.

5 In Figure 14 the composition of plasmid pUR2987 is indicated. Its preparation from plasmid pUR2985 is described in Example 6. It contains a DNA sequence comprising the *choB* gene coding for the mature part of the cholesterol oxidase preceded by DNA encoding the prepro- $\alpha$ -mating factor signal sequence and followed by DNA encoding the C-terminal part of  $\alpha$ -agglutinin.

10 In Figure 15 the composition of the published plasmid pGKV550 is indicated. It is described in Example 7 and contains the complete cell wall proteinase operon of *Lactococcus lactis* subsp. *cremoris* Wg2, including the promoter, the ribosome binding site and the *prtP* gene.

In Figure 16 the composition of plasmid pUR2988 is indicated. Its preparation is described in Example 7. It is anticipated that this plasmid can be used for preparing a further plasmid pUR2989, which after introduction in a lactic acid bacterium will be responsible for producing a chimeric protein that will be anchored at the outer surface of the lactic acid bacterium and is capable of binding cholesterol.

In Figure 17 the composition of plasmid pUR2993 is indicated. Its preparation is described in Example 8. It is anticipated that this plasmid can be used for transforming yeast cells that can bind a human epidermal growth factor (EGF) through an anchored chimeric protein containing an EGF receptor.

In Figure 18 the composition of plasmids pUR4482 and 4483 is indicated. Their preparation is described in Example 9. Plasmid pUR4482 is a yeast episomal expression plasmid for expression of a fusion protein with the invertase signal sequence, the CH<sub>1</sub>09 variable region, the Myc-tail, and the "X-P-X-P" Hinge region of a camel antibody, and the  $\alpha$ -agglutinin cell wall anchor region. Plasmid pUR4483 differs from pUR4482 in that it does not contain the "X-P-X-P" Hinge region.

In Figure 19 immunofluorescent labelling (anti-Myc antibody) of SU10 cells in the exponential phase ( $OD_{530}=0.5$ ) expressing the genes of camel antibodies present on plasmids pUR4424, pUR4482 and pUR4483 is shown.

Ph = phase contrast, Fl = fluorescence.

In Figure 20 immunofluorescent labelling (anti-human IgG antibody) of SU10 cells in the exponential phase ( $OD_{530}=0.5$ ) expressing the genes of camel antibodies present on plasmids pUR4424, pUR4482 and pUR4483 is shown.

Ph = phase contrast, Fl = fluorescence.

5

Abbreviations used in the Figures:

	$\alpha$ -gal:	gene encoding guar $\alpha$ -galactosidase
	AG-alpha-1/AG $\alpha$ 1:	gene expressing $\alpha$ -agglutinin from <i>S. cerevisiae</i>
	AG $\alpha$ 1 cds/ $\alpha$ -AGG:	coding sequence of $\alpha$ -agglutinin
10	Amp/amp r:	$\beta$ -lactamase resistance gene
	CHv09:	camel heavy chain variable 09 fragment
	EmR:	erythromycin resistance gene
	f1:	phage f1 replication sequence
	FLO1/FLO (C-part):	C-terminal part of FLO1 coding sequence of flocculation protein
15	Hinge:	Camel "X-P-X-P" Hinge region, see Example 9
	LEU2:	<i>LEU2</i> gene
	LEU2d/Leu2d:	truncated <i>LEU2</i> gene
	Leu 2d cs:	coding sequence <i>LEU2d</i> gene
20	MycT:	camel Myc-tail
	Ori MB1:	origin of replication MB1 derived from <i>E. coli</i> plasmid
	Pgal7/pGAL7:	<i>GAL7</i> promoter
	Tpgk:	terminator of the phosphoglyceratekinase gene
	pp $\alpha$ -MF/MF $\alpha$ 1ss:	prepro-part of $\alpha$ -mating factor (= signal sequence)
25	repA:	gene encoding the repA protein required for replication (Fig. 15/16).
	ScFv (Vh-Vl):	single chain antibody fragment containing $V_H$ and $V_L$ chains
	ss:	signal sequence
	SUC2:	invertase signal sequence
30	2 $\mu$ m/2 micron:	2 $\mu$ m sequence

**Detailed description of the invention**

The present invention relates to the isolation of valuable compounds from complex mixtures by making use of immobilized ligands. The immobilized ligands can be proteins obtainable via genetic engineering and can consist of two parts, namely

5 both an anchoring protein or functional part thereof and a binding protein or functional part thereof.

The anchoring protein sticks into cell walls of microorganisms, preferably lower eukaryotes, e.g. yeasts and moulds. Often this type of proteins has a long C-terminal 10 part that anchors it in the cell wall. These C-terminal parts have very special amino acid sequences. A typical example is anchoring via C-terminal sequences of proteins enriched in proline, see Kok (1990).

The C-terminal part of these anchoring proteins can contain a substantial number of potential serine and threonine glycosylation sites. O-glycosylation of these sites gives 15 a rod-like conformation to the C-terminal part of these proteins.

In the case of anchored manno-proteins they seem to be linked to the glucan in the cell wall of lower eukaryotes, as they cannot be extracted from the cell wall with sodium dodecyl sulphate (SDS), but can be liberated by glucanase treatment, see our co-pending patent application WO-94/01567 (UNILEVER) published 20 January 20 1994 and Schreuder c.s. (1993), both being published after the claimed priority date. Another mechanism to anchor proteins at the outer side of a cell is to make use of the property that a protein containing a glycosyl-phosphatidyl-inositol (GPI) group anchors via this GPI group to the cell surface, see Conzelmann c.s. (1990).

25 The binding protein is so called, because it ligates or binds to the specific compound to be isolated. If the N-terminal part of the anchoring protein is sufficiently capable of binding to a specific compound, the anchoring protein itself can be used in a process for isolating that specific compound. Suitable examples of a binding protein comprise an antibody, an antibody fragment, a combination of antibody fragments, a 30 receptor protein, an inactivated enzyme still capable of binding the corresponding substrate, and a peptide obtained via Applied Molecular Evolution, see Lewin (1990), as well as a part of any of these proteinaceous substances still capable of

binding to the specific compound to be isolated. All these binding proteins are characterized by specific recognition of the compounds or group of related compounds to be isolated. The binding rate and release rate, and therefore the binding constant between the specific compound to be isolated and the binding protein, can be regulated either by changing the composition of the liquid extract in which the compound is present or, preferably, by changing the binding protein by protein engineering.

The gene coding for the chimeric protein comprising both the binding protein and the anchoring protein (or functional parts thereof) can be placed under control of a constitutive, inducible or derepressible promoter and will generally be preceded by a DNA fragment encoding a signal sequence ensuring efficient secretion of the chimeric protein. Upon secretion the chimeric protein will be anchored in the cell wall of the microorganisms, thereby covering the surface of the microorganisms with the chimeric protein. These microorganisms can be obtained in normal fermentation processes and their isolation is a cheap process, when physical separation processes are used, e.g. centrifugation or membrane filtration.

After washing, the isolated microorganisms can be added to liquid extracts containing the valuable specific compound or compounds. After some time the equilibrium between the bound and free specific compound(s) will be reached and the microorganisms to which the specific compound or group of related compounds is bound can be separated from the extract by simple physical techniques.

Alternatively, the microorganisms covered with ligands can be brought on a support material and subsequently this coated support material can be used in a column.

The liquid extract containing the specific compound or compounds of interest can be added to the column and afterwards the compound(s) can be released from the ligand by changing the composition of the eluting liquid or the temperature or both. A skilled person will recognize that in addition to these two possibilities other modifications can be used for effecting the binding of the specific compound and the ligand, their subsequent isolation and/or the release of the specific compound(s). In particular the invention relates to chimeric proteins that are bound to the cell wall of lower eukaryotes. Suitable lower eukaryotes comprise yeasts, e.g. *Candida*,

*Debaryomyces, Hansenula, Kluyveromyces, Pichia* and *Saccharomyces*, and moulds e.g. *Aspergillus, Penicillium* and *Rhizopus*. For some applications prokaryotes are also applicable, especially Gram-positive bacteria, examples of which include lactic acid bacteria, and bacteria belonging to the genera *Bacillus* and *Streptomyces*.

5

For lower eukaryotes the present invention provides genes encoding chimeric proteins consisting of:

- a. a DNA sequence encoding a signal sequence functional in a lower eukaryotic host, e.g. derived from a yeast protein including the  $\alpha$ -mating factor, invertase, 10  $\alpha$ -agglutinin, inulinase or derived from a mould protein e.g. xylanase;
- b. a structural gene encoding a C-terminal part of a cell wall protein preceded by a structural gene encoding a protein, that is capable of binding to the specific compound or group of compounds of interest, examples of which include
  - an antibody,
  - 15 - a single chain antibody fragment (scFv; see Bird and Webb Walker (1991),
    - a variable region of the heavy chain ( $V_H$ ) or a variable region of the light chain ( $V_L$ ) of an antibody or that part of such variable region still containing one to three of the complementarity determining regions (CDRs),
    - an agonist-recognizing part of a receptor protein or a part thereof still capable 20 of binding the agonist,
    - a catalytically inactivated enzyme, or a fragment of such enzyme still containing a substrate binding site of the enzyme,
    - specific lipid binding proteins or parts of these proteins still containing the lipid binding site(s), see Ossendorp (1992), and
  - 25 - a peptide that has been obtained via Applied Molecular Evolution, see Lewin (1990).

All expression products of these genes are characterized in that they consists of a signal sequence and both a protein part, that is capable of binding to the compound(s) to be isolated, and a C-terminus of a typically cell wall bound protein, 30 examples of the latter including  $\alpha$ -agglutinin, see Lipke c.s. (1989),  $\alpha$ -agglutinin, see Roy c.s. (1991), FLO1 (see Example 5 and SEQ ID NO: 14) and the Major Cell

Wall Protein of lower eukaryotes, which C-terminus is capable of anchoring the expression product in the cell wall of the lower eukaryote host organism.

The expression of these genes encoding chimeric proteins can be under control of a constitutive promoter, but an inducible promoter is preferred, suitable examples of

5 which include the GAL7 promoter from *Saccharomyces*, the inulinase promoter from *Kluyveromyces*, the methanol-oxidase promoter from *Hansenula*, and the xylanase promoter of *Aspergillus*. Preferably the constructs are made in such a way that the new genetic information is integrated in a stable way in the chromosome of the host cell, see e.g. WO-91/00920 (UNILEVER).

10 The lower eukaryotes transformed with the above mentioned genes can be grown in normal fermentation, continuous fermentation, or fed batch fermentation processes. The selection of a suitable process for growing the microorganism will depend on the construction of the gene and the promoter used, and on the desired purity of the cells after the physical separation procedure(s).

15 For bacteria the present invention deals with genes encoding chimeric proteins consisting of:

a. a DNA sequence encoding a signal sequence functional in the specific bacterium, e.g. derived from a *Bacillus*  $\alpha$ -amylase, a *Bacillus subtilis* subtilisin, or a

20 *Lactococcus lactis* subsp. *cremoris* proteinase;

b. a structural gene encoding a C-terminal part of a cell wall protein preceded by a structural gene encoding a protein capable of binding to the specific compound or group of compounds of interest, examples of which are given above for a lower eukaryote.

25 All expression products of these genes are characterized in that they consist of a signal sequence and both a protein part, that is capable of binding to the specific compound or specific group of compounds to be isolated, and a C-terminus of a typically cell wall-bound protein such as the proteinase of *Lactococcus lactis* subsp. *cremoris* strain Wg2, see Kok c.s. (1988) and Kok (1990), the C-terminus of which is

30 capable of anchoring the expression product in the cell wall of the host bacterium.

The invention is illustrated with the following Examples without being limited thereto. First the endonuclease restriction sites mentioned in the Examples are given.

5	<i>Bst</i> III	G GTNACC CCANTG G	<i>Cla</i> I	AT CGAT TAGC TA	<i>Eag</i> I	C GGCG GCCGG C
10	<i>Eco</i> RI	G AATTC CTTAA G	<i>Hind</i> III	A AGCTT TTCCGA A	<i>Nhe</i> I	G CTAGC CGATC G
	<i>Not</i> I	GC GGCCGC CGCCGG CG	<i>Nru</i> I	TCG CGA AGC GCT	<i>Pst</i> I	CTGCA G G ACGTC
15	<i>Sac</i> I	GAGCT C C TCGAG	<i>Sal</i> I	G TCGAC CAGCT G	<i>Xba</i> I	C TCGAG GAGCT C

Example 1. Construction of a gene encoding a chimeric protein that will be  
20 anchored in the cell wall of a lower eukaryote and is able to bind  
with high specificity lysozyme from a complex mixture.

Lysozyme is an anti-microbial enzyme with a number of applications in the pharmaceutical and food industries. Several sources of lysozyme are known, e.g. egg yolk or a fermentation broth containing a microorganism producing lysozyme.  
25 Monoclonal antibodies have been raised against lysozyme, see Ward *et al.* (1989), and the mRNA's encoding the light and heavy chains of such antibodies have been isolated from the hybridoma cells and used as template for the synthesis of cDNA using reverse transcriptase. Starting from the plasmids as described by Ward *et al.* (1989), we constructed a pEMBL-derived plasmid, designated pUR4122, in which  
30 the multiple cloning site of the pEMBL-vector, ranging from the *Eco*RI to the *Hind*III site, was replaced by a 231 bp DNA fragment, whose nucleotide sequence is given in SEQ ID NO: 1 and has an *Eco*RI site (GAATTC) at nucleotides 1-6, a *Pst*I site (CTGCAG) at nucleotides 105-110, a *Bst*III site (GGTCACC) at nucleotides 122-128, a *Xba*I site (CTCGAG) at nucleotides 207-212, and a *Hind*III site  
35 (AAGCTT) at nucleotides 226-231.

Construction of pUR4122

Plasmid pEMBL9, see Dente c.s. (1983), was digested with *Eco*RI and *Hind*III and the resulting large fragment was ligated with the double stranded synthetic DNA fragment given in SEQ ID NO: 1. For the successive ligation of DNA fragments, 5 which finally form the coding sequence of a single chain antibody fragment for lysozyme, the following elements were combined in the 231 bp DNA fragment (SEQ ID NO: 1) inserted into the pEMBL-9 vector: the 3' part of the *GAL7* promoter, the invertase signal sequence (SUC2), a *Pst*I restriction site, a *Bst*EII restriction site, a sequence encoding the (GGGGS)x3 peptide linker connecting the  $V_H$  and  $V_L$  frag- 10 ments, a *Sac*I restriction site, a *Xba*I restriction site and a *Hind*III restriction site, resulting in plasmid pUR4119. To obtain the in frame fusion between  $V_H$  and the GGGGS-linker plasmid pSW1-VHD1.3-VKD1.3-TAG1, see Ward c.s. (1989), was digested with *Pst*I and *Bst*EII and a DNA fragment of 0.35 kbp was ligated in the correspondingly digested pUR4119 resulting in plasmid pUR4119A. Subsequently 15 the plasmid pSW1-VHD1.3-VKD1.3-TAG1 was digested with *Sac*I and *Xba*I and this fragment containing the coding part of  $V_L$  was finally ligated into the *Sac*I/*Xba*I sites of pUR4119A, resulting in plasmid pUR4122 (see Figure 1).

Construction of pUR4174, see Figure 4

20 To obtain *S. cerevisiae* episomal expression plasmids containing DNA encoding a cell wall anchor derived from the C-terminal part of  $\alpha$ -agglutinin, plasmid pUR2741 (see Figure 2) was selected as starting vector. Basically, this plasmid is a derivative of pUR2740, which is a derivative of plasmid pUR2730 as described in WO-91/19782 (UNILEVER) and by Verbakel (1991). The preparation of pUR2730 is clearly 25 described in Example 9 of EP-A1-0255153 (UNILEVER). Plasmid pUR2741 differs from plasmid pUR2740 in that the *Eag*I restriction site within the remaining part of the already inactive *tet* resistance gene was deleted through *Nru*I/*Sal*I digestion. The *Sal*I site was filled in prior to religation.

30 After digesting pUR4122 with *Sac*I (partially) and *Hind*III, the approximately 800 bp fragment was isolated and cloned into the pUR2741 vector fragment, which was

obtained after digestion of pUR2741 with the same enzymes. The resulting plasmid was named pUR4125.

A plasmid named pUR2968 (see Figure 3) was made by (1) digesting with *Hind*III the *Agα1*-containing plasmid pLα21 published by Lipke c.s. (1989), (2) isolating an 5 about 6.1 kbp fragment and (3) ligating that fragment with *Hind*III-treated pEMBL9, so that the 6.1 kbp fragment was introduced into the *Hind*III site present in the multiple cloning site of the pEMBL9 vector.

Plasmid pUR4125 was digested with *Xba*I and *Hind*III and the about 8 kbp fragment was ligated with the approximately 1.4 kbp *Nhe*I-*Hind*III fragment of 10 pUR2968, using *Xba*I/*Nhe*I adapters having the following sequence:

<i>Xba</i> I	<i>Nhe</i> I	
5'- <u>TC GAG</u> ATC AAA GGC GGA TCT <u>G</u> -3'		= SEQ ID NO: 2
3'- <u>C</u> TAG TTT CCG CCT AGA <u>CGATC</u> -5'		= SEQ ID NO: 3.

The plasmid resulting from the ligation of the appropriate parts of plasmids 15 pUR2968, pUR4125 and *Xba*I/*Nhe*I adapters, was designated pUR4174 and encodes a chimeric fusion protein at the amino terminus consisting of the invertase signal (pre) peptide, followed by the scFv-LYS polypeptide and, finally, the C-terminal part of  $\alpha$ -agglutinin (see Figure 4).

20 Construction of pUR4175, see Figure 4

Upon digesting pUR4122 (see above) with *Pst*I and *Hind*III, the approximately 700 bp fragment was isolated and ligated into a vector fragment of plasmid pSY16; see Harmsen c.s. (1993), which was digested with *Eag*I and *Hind*III and using *Eag*I-*Pst*I adapters, having the following sequence:

<i>Eag</i> I	<i>Pst</i> I	
5'- <u>G</u> <u>GCC</u> <u>GCC</u> CAG GTG CAG <u>CTG CA</u> -3'		= SEQ ID NO: 4
3'- <u>CGG</u> GTC CAC GTC <u>G</u> -5'		= SEQ ID NO: 5

The resulting plasmid, named pUR4132, was digested with *Xba*I and *Hind*III and ligated with the approximately 1.4 kbp *Nhe*I-*Hind*III fragment of pUR2968 (see 30 above), using *Xba*I/*Nhe*I adapters as described above, resulting in pUR4175 (see Figure 4). This plasmid contains a gene encoding a chimeric protein consisting of the  $\alpha$ -mating factor prepro-peptide, followed by the scFv-LYS polypeptide and, finally, the C-terminal part of  $\alpha$ -agglutinin.

**Example 2.** Construction of genes encoding a series of homologous chimeric proteins that will be anchored in the cell wall of a lower eukaryote and are able to bind with high specificities the musk fragrance trascolide® from a complex mixture.

5 The isolation of RNA from the hybridoma cell lines, the preparation of cDNA and amplification of gene fragments encoding the variable regions of antibodies by PCR was performed according to standard procedures known from the literature, see e.g. Orlandi *et al.* (1989). For the PCR amplification different oligonucleotide primers have been used.

10 For the heavy chain fragment:

A: AGG TSM ARC TGC AGS AGT CWG G = SEQ ID NO: 6  
*PstI*

in which S is C or G, M is A or C, R is A or G, and W is A or T, and

15 B: TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC = SEQ ID NO: 7.  
*BstEII*

For the light chain fragment (Kappa):

C: GAC ATT GAG CTC ACC CAG TCT CCA = SEQ ID NO: 8,  
*SacI*

20 and

D: GTT TGA TCT CGA GCT TGG TCC C = SEQ ID NO: 9.  
*XbaI*

#### Construction of pUR4143

25 To simplify future construction work an *EagI* restriction site was introduced in pUR4122 (see above), at the junction between the invertase signal sequence and the scFv-LYS. This was achieved by replacing the about 110 bp *EcoRI-PstI* fragment within the synthetic fragment given in SEQ ID NO: 1 by synthetic adapters with the following sequence:

30 *EcoRI* *PstI*  
AATTGGCCGTTCAAGTGCAGCTGCA = SEQ ID NO: 10  
GCGGGCAAGTCCACGTCG = SEQ ID NO: 11.

The resulting plasmid was designated pUR4122.1: a construction vector for single chain Fv assembly in frame behind an *EagI* site for expression behind either the prepro- $\alpha$ -mating factor sequence or the SUC2 invertase signal sequence.

After digesting the heavy chain PCR fragment with *PstI* and *BstEII*, two fragments 5 were obtained: a *PstI* fragment of about 230 bp and a *PstI/BstEII* fragment of about 110 bp. The latter fragment was cloned into vector pUR4122.1, which was digested with *PstI* and *BstEII*. The newly obtained plasmid (pUR4122.2) was digested with *SacI* and *Xhol*, after which the light chain PCR fragment (digested with the same restriction enzymes) was cloned into the vector, resulting in pUR4122.3. This 10 plasmid was digested with *PstI*, after which the above described about 230 bp *PstI* fragment was cloned into the plasmid vector, resulting in a plasmid called pUR4143. Two orientations are possible, but selection can be made by restriction analysis, as usual. Instead of the scFv-LYS gene originally present in pUR4122, this new plasmid pUR4143 contains a gene encoding an scFv-TRAS fragment of anti-trasolide 15 antibody 02/01/01 (for the nucleotide sequence of the 714 bp *PstI-Xhol* fragment see SEQ ID NO: 12).

Construction of pUR4178 and pUR4179.

After digesting pUR4143 with *EagI* and with *HindIII*, an about 715 bp fragment can 20 be isolated. Subsequently, this fragment can be cloned into the vector backbone fragments of pUR2741 and pUR4175, that were digested with the same restriction enzymes. In the case of pUR2741, this resulted in plasmid pUR2743.4 (see Figure 5). This plasmid can subsequently be cleaved with *Xhol* and *HindIII* and ligated with the about 8 kbp *Xhol-HindIII* fragment of pUR4174, resulting in pUR4178 (see 25 Figure 6).

In the situation where pUR4175 was used as a starting vector, the resulting plasmid was designated pUR4179 (see Figure 7).

Both plasmids, pUR4178 and pUR4179 were introduced into *S. cerevisiae*.

**Example 3.** The modification of the binding parts of the chimeric protein that can bind traseolide® in order to improve the binding or release of traseolide® under certain conditions.

Modification of binding properties of antibodies during the immune response is a well known immunological phenomenon originating from the fine tuning of complementarity determining sequences in the antibody's binding region to the antigen's molecular properties. This phenomenon can be mimicked *in vitro* by adjusting the antigen binding regions of antibody fragments based on molecular models of these regions in contact with the antigen.

One such example consists of protein engineering the antimusk antibody M02/01/01 to a stronger binding variant M020501i.

First, a molecular model of M02/01/01 variable fragment (Fv) was constructed by homology modelling, using the coordinates of the anti-lysozyme antibody HYHEL-10 as a template (Brookhaven Protein Data Bank entry: 3HFM). This model was refined using Molecular Mechanics and Molecular Dynamics methods from within the Biosym program DISCOVER, on a Silicon Graphics 4D240 workstation.

Secondly, the binding site of the resulting Fv was mapped by visually docking the musk antigen into the CDR region, followed by a refinement using molecular dynamics again. Upon inspection of the resulting model for packing efficiency (van der Waals contact areas), it was concluded that substitution of ALA H96 by VAL would increase the (hydrophobic) contact area between the ligand and Fv, and consequently lead to a stronger interaction (see Figure 8).

When this mutation is introduced into M02/01/01, the cDNA-derived scFv from Example 2, the result will be Fv M020501i; a variant with an increased affinity of at least a factor of 5 can be expected, and the increased affinity could be measured using fluorescence titration of the Fv with the musk odour molecule.

**Example 4.** Construction of a gene encoding a chimeric protein that will be anchored in the cell wall of lower eukaryote and is able to bind hormones such as HCG.

Gene fragments, encoding the variable regions of the heavy and light chain fragments from the monoclonal antibody directed against the human chorionic gonadotropin were obtained from a hybridoma cell line in a similar way as described in Example 2.

Subsequently, these HCG  $V_{H}$  and  $V_{L}$  gene fragments were cloned into plasmid pUR4143 by replacing the corresponding *PstI-Bst*Ell and *SacI-Xba*I gene fragments, 10 resulting in plasmid pUR4146.

Similar to the method described in Example 2, the 734 bp *EagI-Xba*I fragment (nucleotide sequence given in SEQ ID NO: 13) encoding the variable regions of the heavy and light chain fragments from the monoclonal antibody directed against the human chorionic gonadotropin (an scFv-HCG fragment) was isolated from pUR4146 15 and was introduced into the vector backbone fragment of pUR4178 (see Example 2) and will be introduced into the vector backbone fragment of pUR4175 (see Example 1), both digested with the same restriction enzymes. The resulting plasmids pUR4177 (see Figure 9) was, and pUR4180 (see Figure 10) will be, introduced into *S. cerevisiae* strain SU10.

20

**Example 5.** Construction of a gene encoding a chimeric scFv-FLO1 protein that will be anchored in the cell wall of lower eukaryote and is able to bind hormones such as HCG.

25 One of the genes associated with the flocculation phenotype in *S. cerevisiae* is the FLO1 gene. The DNA sequence of a clone containing major parts of the FLO1 gene has been determined, see SEQ ID NO: 14 giving 2685 bp of the FLO1 gene. The cloned fragment appeared to be approximately 2 kb shorter than the genomic copy as judged from Southern and Northern hybridizations, but encloses both ends of the 30 FLO1 gene. Analysis of the DNA sequence data indicates that the putative protein contains at the N-terminus a hydrophobic region which confirms a signal sequence for secretion, a hydrophobic C-terminus that might function as a signal for the

attachment of a GPI-anchor and many glycosylation sites, especially in the C-terminus, with 46.6% serine and threonine in the arbitrarily defined C-terminus (aa 271-894). Hence, it is likely that the FLO1 gene product is located in an orientated fashion in the yeast cell wall and may be directly involved in the process 5 of interaction with neighbouring cells.

The cloned FLO1 sequence might therefore be suitable for the immobilization of proteins or peptides on the cell surface by a different type of cell wall anchor.

For the production of a chimeric protein comprising the scFv-HCG followed by the C-terminal part of the FLO1-protein, plasmid pUR2990 (see Figure 11) can be used 10 as a starting vector. The preparation of episomal plasmid pUR2990 was described in our co-pending patent application WO-94/01567 (UNILEVER) published on 20 January 1994, i.e. during the priority year. Plasmid pUR2990 comprises the chimeric gene consisting of the gene encoding the *Humicola* lipase and a gene encoding the putative C-terminal cell wall anchor domain of the FLO1 gene product, the chimeric 15 gene being preceded by the invertase signal sequence (SUC2) and the GAL7 promoter; further the plasmid comprises the yeast 2  $\mu$ m sequence, the defective Leu2 promoter described by Eckard and Hollenberg (1983), and the Leu2 gene, see Roy c.s. (1991). Plasmid pUR4146, described in Example 4, can be digested with *Pst*I and *Xho*I, and the about 0.7 kbp *Pst*I-*Xho*I fragment containing the scFv-HCG 20 coding sequence can be isolated. For the in frame fusion of this DNA sequence between the C-terminal FLO1 part and the SUC2 signal sequence, the fragment can be directly ligated with the 9.3 kbp *Eag*I/*Nhe*I (partial) backbone of plasmid pUR2990, resulting in plasmid pUR4196 (see Figure 12). This plasmid will comprise an additional triplet encoding Ala at the transition between the SUC2 signal 25 sequence and the start of the scFv-HCG, and a E-I-K-G-G amino acid sequence in front of the first amino acid (Ser) of the C part of FLO1 protein.

If in the previous Examples 1-5 the level of exposed antibody fragments is too low, the production level can be increased by mutagenesis of the frame work regions of 30 the antibody fragment. This can be done in a site directed way or by (targeted) random mutagenesis, using techniques described in the literature.

**Example 6.** Construction of a gene encoding a chimeric protein that will be anchored in the cell wall of a lower eukaryote and is able to bind cholesterol.

In the literature two DNA sequences for cholesterol oxidase are described, the *choB* gene from *Brevibacterium sterolicum*, see Ohta c.s. (1991) and the *choA* gene from *Streptomyces sp.* SA-COO, see Ishizaka c.s. (1989). For the construction of a DNA fusion between the *choB* gene coding for cholesterol oxidase (EC 1.1.3.6) and the 3' part of the  $\alpha$ -1 gene, the PCR technique on chromosomal DNA can be applied. Chromosomal DNA can be isolated by standard techniques from 5 *Brevibacterium sterolicum*, and the DNA part coding for the mature part of the cholesterol oxidase can be amplified through application with the following 10 corresponding PCR primers *cho01pcr* and *cho02pcr*:

**cho01pcr**

15	5'	GCC CCC AGC CGC ACC CTC G-3'	= SEQ ID NO: 16	
	3'	CGG GGG TCG GCG TGG GAG C-5'	= SEQ ID NO: 17	
	5'	AGATCT <u>GAATT</u> CGCGGCC	GCC CCC AGC CGC ACC CTC G-3'	= SEQ ID NO: 18
20		EcoRI NotI		
		EagI		

**cho02pcr**

25	3'	TAG TAG AGC AGG CTG TAG GTC <u>CGATCGACTTTCGAATCTAGA</u> -5'	= SEQ ID NO: 19
			= SEQ ID NO: 20
	5'	ATC ATC TCG TCC GAC ATC CAG-3'	= SEQ ID NO: 21
	3'	TAG TAG AGC AGG CTG TAG GTC-5'	

Both primers can specifically hybridize with the target sequence, thereby amplifying the coding part of the gene in such a way, that the specific PCR product -after 30 Proteinase K treatment and digestion with *Eco*RI and *Hind*III- can be directly cloned into a suitable vector, here preferably pTZ19R, see Mead c.s. (1986). This will result in plasmid pUR2985 (see Figure 13).

In addition to the already mentioned restriction sites both PCR primers generate other restriction sites at the 5' end and the 3' end of the 1.5 kbp DNA fragment, 35 which can be used later on to fuse the fragment in frame between either the *SUC2* signal sequence or the prepro- $\alpha$ -mating factor signal sequence on one side and the C-terminus coding part of the  $\alpha$ -agglutinin gene on the other side. To facilitate the ligation behind the prepro-MF sequence a *Not*I site is introduced at the 5' end of

PCR oligonucleotide cho01pcr, allowing for example, the exchange of the 731 bp *EagI/NheI* fragment containing the scFv-Lys coding sequence in pUR4175 for the *choB* coding sequence.

To create an enzymatically inactive fusion protein between cholesterol oxidase and 5  $\alpha$ -agglutinin, the above described subcloning into pTZ19R can be used. Cholesterol oxidase is an FAD-dependent enzyme for which the crystal structure of the *Brevibacterium sterolicum* enzyme has been determined, see Vrielink c.s. (1991). The enzyme displays homology with the typical pattern of the FAD-binding domain with the Gly-X-Gly-X-X-Gly sequence near the N-terminus (amino acid 18-23). Site- 10 directed *in vitro* mutagenesis on the plasmid pUR2985 according to the manufacturer's protocol (Muta-Gene kit, Bio-Rad) can be applied to inactivate the FAD-binding site through replacing the triplet(s) encoding the Gly residue(s) by triplets encoding other amino acids, thereby presumably inactivating the enzyme. E.g. the following primer can be used for site-directed mutagenesis of 2 of the 15 conserved Gly residues.

20 pr 3' - CGG GAG CAG TAG CGG TCA CGT ATG CCG CCA CGG CAG CGG CGC -5'  
cs 5' - GCC CTC GTC ATC GCC AGT GGA TAC GGC GGT GCC GTC GCC GCG -3'  
Ala Gly Gly Gly Ala Ala Ala  
↓ ↓  
Ala Ala

pr = primer = SEQ ID NO: 22  
cs = coding strand = SEQ ID NO: 23

25 As a result of the mutagenesis with the described primer, plasmid pUR2986 will be obtained. From this plasmid the DNA coding for the presumably inactivated cholesterol oxidase can be released as a 1527 bp fragment through *NotI/NheI* digestion, and subsequently directly used to exchange the scFv-Lys coding sequence 30 in pUR4175, thereby generating plasmid pUR2987 (see Figure 14). To obtain a variant yeast secretion vector, where the secretion is directed through the *SUC2* signal sequence, for example the 1823 bp long *SacI/NheI* segment of plasmid pUR2986 can be used to replace the *SacI/NheI* fragment in pUR4174. This inactivation of the FAD-binding site might be preferable over other mutations, 35 since an unchanged active centre can be expected to leave the binding properties of cholesterol oxidase for cholesterol unaltered. Instead of the described Gly-Ala

exchanges at position 18 and 20 of the mature coding sequence, every other suitable amino acid change can also be performed.

To inactivate the enzyme, site directed mutagenesis can be optionally immediately performed in the active site cavity, for example through exchange of the Glu331, a 5 residue appropriately positioned to act as the proton acceptor, thus generating a new variant of an immobilized, enzymatically inactive fusion protein.

10 **Example 7. Construction of a gene encoding a chimeric protein that will be anchored in the cell wall of a lactic acid bacterium and is able to bind cholesterol.**

15 It has been described that proteinase of *Lactococcus lactis* subsp. *cremoris* is anchored to the cell wall through its 127 amino acid long C-terminal, see Kok *et al.* (1988) and Kok (1990). In a way similar to that described in Example 6, the cholesterol oxidase of *Brevibacterium sterolicum* (*choB*) can be immobilized on the 20 surface of *Lactococcus lactis*. Fusions can be made can be made between the *choB* structural gene and the N-terminal signal sequence and the C-terminal anchor of the proteinase of *Lactococcus lactis*. Plasmid pGKV550 (see Figure 15) contains the complete proteinase operon of *Lactococcus lactis* subsp. *cremoris* Wg2, including the promoter, a ribosome binding site and DNA fragments encoding the already mentioned signal and anchor sequences, see Kok (1990). First a DNA fragment, containing the main part of the signal sequence, flanked by a *Cla*I site and an *Eag*I site can be constructed with PCR on pGKV550 as follows:

25 **Primer prt1:**

5'-AA GAT CTA TCG ATC TTG TTA GCC GGT ACA-3' = SEQ ID NO: 24

Proteinase gene (non coding strand):

3'-TT CCC GAT AGC TAG AAC AAT CGG CCA TGT CAG-5'  
*Cla*I = SEQ ID NO: 25

30

Proteinase gene: Gln Ala Lys

5'-GTC GGC GAA ATC CAA GCA AAG GCG GCT-3' = SEQ ID NO: 26

Primer prt2: = SEQ ID NO: 27

35

3'-CAG CCG CTT TAG GTT CGT TGC CGG CCC CCC TTC GAA CCC-5'  
*Eag*I *Hind*III

After the PCR reaction as described in Example 6, the 98 bp long PCR fragment can be isolated and digested with *Clal* and *Hind*III. pGKV550 can subsequently be cleaved partially with *Clal* and completely with *Hind*III, after which digestions the vector fragment, containing the promoter, the ribosome binding site, the DNA

5 fragment encoding the N-terminal 8 amino acids and the cell wall binding fragment containing the 127 C-terminal amino acids of the proteinase gene can be isolated on gel.

A copy of the cholesterol oxidase gene, suitable for fusion with the *prtP* anchor domain can be produced by a PCR reaction using plasmid pUR2985 (Example 6) as template and a combination of primer *cho01pcr* (see Example 6) and the following primer *cho03pcr* instead of primer *cho02pcr*:

cho03pcr *HindIII*  
 15 3'-TAG TAG AGC AGG CTG TAG GTC CGA GTT CGA ACC TAG GC-5' = SEQ ID NO: 40  
       ||| ||| ||| ||| ||| ||| |||  
 5'-ATC ATC TCG TCC GAC ATC CAG = SEQ ID NO: 20.

The about 1.53 kbp fragment generated by this reaction can be digested with *Not*I and *Hind*III to produce a molecule which can subsequently be ligated with the large *Eag*I/*Hind*III fragment from pUR2988 (see Figure 16). The resulting plasmid,

20 pUR2989, will contain the cholesterol oxidase coding sequence inserted between the signal sequence and the C-terminal cell wall anchor domain of the proteinase gene. After introduction into *Lactobacillus lactis* subsp. *lactis* MG1363 by electroporation, this plasmid will express cholesterol oxidase under control of the proteinase promoter. The transport through the membrane will be mediated by the proteinase 25 signal sequence and the immobilization of the cholesterol oxidase by the proteinase anchor. As it is unlikely that the *Lactococcus* will secrete FAD as well, the cholesterol oxidase will not be active but will be capable to bind cholesterol.

30 Example 8. Construction of a gene encoding a chimeric protein that will be anchored in the cell wall of a lower eukaryote and is able to bind growth hormones, such as the epidermal growth factor.

For the isolation of larger amounts of human epidermal growth factor (EGF) the corresponding receptor can be used in form of a fusion between the binding domain

and a C-terminal part of  $\alpha$ -agglutinin as cell wall anchor. The complete cDNA sequence of the human epidermal growth factor is cloned and sequenced. For the construction of a fusion protein with EGF binding capacity the N-terminal part of the mature receptor until the central 23 amino acids transmenbrane region can be 5 utilized.

The plasmid pUR4175 can be used for the construction. Through digestion with *EagI* and *NheI* (partial) a 731 bp DNA fragment containing the sequence coding for scFv is released and can be replaced by a DNA fragment coding for the first 621 amino acids of human epidermal growth factor receptor. Initiating from an existing 10 human cDNA library or otherwise through production of a cDNA library by standard techniques from preferentially EGF receptor overexpressing cells, e.g. A431 carcinoma cells, see Ullrich c.s. (1984), further PCR can be applied for the generation of in frame linkage between the extracellular binding domain of the 15 human growth factor receptor (amino acid 1-622) and the C-terminal part of  $\alpha$ -agglutinin.

PCR oligonucleotides for the in frame linkage of human epidermal growth factor receptor and the C-terminus of  $\alpha$ -agglutinin.

20 a: PCR oligonucleotides for the transition between SUC2 signal sequence and the N-terminus of mature EGF receptor.

25 >mature EGF receptor  
pri EGF1: Ala Leu Glu Lys Lys Val = SEQ ID NO: 28  
5'-GGG GCG GCC GCG CTG GAG GAA AAG AAA GTT TGC-3'  
Not1 ||||| ||||| ||||| ||||| ||||| |||||  
3'-CGC TCA GCC CGA GAC CTC CTT TTC TTT CAA ACG 5'  
EGF rec (non-coding strand): = SEQ ID NO: 29

b: PCR oligonucleotides for the in frame transition between C terminus of the 30 extracellular binding domain of EGF receptor and the C terminal part of  $\alpha$ -agglutinin.

## EGF rec (coding strand):

Asn Gly Pro Ile Pro Ser Ala Thr  
5'-AAT GGG CCT AAG ATC CCG TCC ATC GCC ACT-3' = SEQ ID NO: 30  
||| ||| ||| ||| ||| ||| ||| |||  
5 3'-TTA CCC GGA TTC TAG GGC AGG CGA TCGGAATTGAA CCCC-5'  
pr EGF2: NheI HindIII

This fusion would result in an addition of 2 Ala amino acids between the signal sequence and the mature N-terminus of EGF receptor.

The newly obtained 1.9 kbp PCR fragment can be digested with *Not*I and *Nhe*I and 10 directly ligated into the vector pUR4175 after digesting with the same enzymes, resulting in plasmid pUR2993 (see Figure 17), comprising the *GAL7* promoter, the prepro- $\alpha$ -mating factor sequence, the chimeric EGF receptor binding domain gene /  $\alpha$ -agglutinin gene, the yeast 2  $\mu$ m sequence, the defective *LEU2* promoter and the *LEU2* gene. This plasmid can be transformed into *S. cerevisiae* and the transformed 15 cells can be cultivated in YP medium whereby expression of the chimeric protein can be induced by adding galactose to the medium.

20 **Example 9. Construction of genes encoding a chimeric protein anchored to the cell wall of yeast, comprising a binding domain of a "Camelidae" heavy chain antibody**

Recently it was described that camels as well as a number of related species (e.g. 25 lamas) contain a considerable amount of IgG antibody molecules which are only composed of heavy-chain dimers, see Hamers-Casterman *et al.* (1993). Although these "heavy-chain" antibodies are devoid of light chains, it was demonstrated, that they nevertheless have an extensive antigen-binding repertoire. In order to show that the variable regions of this type of antibodies can be produced and will be linked to the exterior of the cell wall of a yeast, the following constructs were prepared.

30 **Construction of pUR2997, pUR2998 and pUR2999**

The about 2.1 kbp *Eag*I-*Hind*III fragment of pUR4177 (Example 4, Fig 9) was isolated. By using PCR technology, an *Eco*RI restriction site was introduced immediately upstream of the *Eag*I site, whereby the C of the *Eco*RI site is the same as the first C of the *Eag*I site. The thus obtained *Eco*RI-*Hind*III fragment was

ligated into plasmid pEMBL9, which was digested with *Eco*RI and *Hind*III, which resulted in pUR4177.A

The *Eco*RI/*Nhe*I fragment of plasmid pUR4177.A was replaced by the *Eco*RI/*Nhe*I fragments of three different synthetic DNA fragments (SEQ ID NO: 32, SEQ ID

5 NO: 33, and SEQ ID NO: 34) resulting in pUR2997, pUR2998 and pUR2999, respectively. The about 1.5 kbp *Bst*EII-*Hind*III fragments of pUR2997 and pUR2998 were isolated.

Construction of pUR4421

10 The multiple cloning site of plasmid pEMBL9, see Dente c.s. (1983), (ranging from the *Eco*RI to the *Hind*III site) was replaced by a synthetic DNA fragment having the nucleotide sequence given below, see SEQ ID NO: 35 giving the coding strand and SEQ ID NO: 36 giving the non-coding strand. The 5'-part of this nucleotide sequence comprises an *Eag*I site, the first 4 codons of a *Camelidae V<sub>H</sub>* gene

15 fragment (nucleotides 16-27) and a *Xho*I site (CTCGAG) coinciding with codons 5 and 6 (nucleotides 28-33). The 3'-part comprises the last 5 codons of the *Camelidae V<sub>H</sub>* gene (nucleotides 46-60) (part of which coincides with a *Bst*EII site), eleven codons of the Myc tail (nucleotides 61-93), see SEQ ID NO: 35 containing these eleven codons and SEQ ID NO: 37 giving the amino acid sequence, and an *Eco*RI

20 site (GAATTC). The *Eco*RI site, originally present in pEMBL9, is not functional any more, because the 5'-end of the nucleotide sequence contains AATTTC instead of AATTC, indicated below as (*Eco*RI). The resulting plasmid is called pUR4421.

The *Camelidae V<sub>H</sub>* fragment starts with amino acids Q-V-K and ends with amino acids V-S-S.

25	( <i>Eco</i> RI) <i>Eag</i> I	<i>Xho</i> I	<i>Bst</i> EII	
	5'- <u>AATTTCAGCGG</u> CCGCCCAGGT GAAACTGCTC <u>GAGTAAGTGA</u> CTAAGGTAC-			50
	3' 1 ATCGCC GGCGGGTCCA CTTTGACGAG CTCATTCACT GATTCCAGTG-			
	5 Q V K			
30	-CGTCTCCTCA GAACAAAAAC TCATCTCAGA AGAGGGATCTG <u>AATTAATGAG</u> - 100			
	-GCAGAGGAGT CTTGTTTTG AGTAGAGTCT TCTCCTAGAC TTAATTACTC-			
	V S S E Q K L I S E E D L N * *	= SEQ ID NO: 37		
35	<i>Eco</i> RI	<i>Hind</i> III		
	-AATTCAACCGGTGATA	-3'	119	= SEQ ID NO: 35
	-TTAAGTAGTT TGCCACTATT CGA	-5'	123	= SEQ ID NO: 36

(1993), and the  $\alpha$ -agglutinin cell wall anchor region. Plasmid pUR4483 differs from pUR4482 in that it contains the Myc-tail but not the "X-P-X-P" Hinge region. Similarly, the *Bst*ECII-*Hind*III fragment from pUR2999 can be ligated with the about 6.3 kbp vector fragment and the about 0.44 kbp fragment from pUR4424, resulting 5 in pUR4497, which will differ from pUR4482 in that it contains the "X-P-X-P" Hinge region but not the Myc-tail.

The plasmids pUR4424, pUR4482 and pUR4483 were introduced into *Saccharomyces cerevisiae* SU10 by electroporation, and transformants were selected on plates lacking leucine. Transformants from SU10 with pUR4424, pUR4482 or 10 pUR4483, respectively, were grown on YP with 5% galactose and analysed with immuno-fluorescence microscopy, as described in Example 1 of our co-pending WO-94/01567 (UNILEVER) published on 20 January 1994. This method was slightly modified to detect the chimeric proteins, containing both the camel antibody and the Myc tail, present at the cell surface.

15 In one method a monoclonal mouse anti-Myc antibody was used as a first antibody to bind to the Myc part of the chimeric protein; subsequently a polyclonal anti-mouse Ig antiserum labeled with fluorescein isothiocyanate (= FITC) ex Sigma, Product No. F-0527, was used to detect the bound mouse antibody and a positive signal was determined by fluorescence microscopy.

20 In the other method a polyclonal rabbit anti-human IgG serum, which had earlier been proven to cross-react with the camel antibodies, was used as a first antibody to bind the camel antibody part of the chimeric protein; subsequently a polyclonal anti-rabbit Ig antiserum labeled with FITC ex Sigma, Product No. F-0382, was used to detect the bound rabbit antibody and a positive signal was determined by 25 fluorescence microscopy.

The results in Figure 19 and Figure 20 show clearly that fluorescence can be observed on those cells in which a fusion protein of the CH<sub>v</sub>09 fragment with the  $\alpha$ -agglutinin cell wall anchor region is produced (pUR4482 and pUR4483). No 30 fluorescence however, was visible on the cells which produce the CH<sub>v</sub>09 fragment without this anchor (pUR4424), when viewed under the same circumstances.

Patent literature references:

EP-A1-0255153 (UNILEVER) Production of guar alpha-galactosidase by hosts transformed by recombinant DNA methods. First priority date 03.06.86; published 03.02.88

5 WO-91/00920 (UNILEVER) Process for preparing a protein by a fungus transformed by multicopy integration of an expression vector. First priority date 07.07.89; published 24.01.91

WO-91/19782 (UNILEVER) Xylanase production. Priority date 19.06.90; published 26.12.91

10 WO-94/01567 (UNILEVER) Process for immobilizing enzymes to the cell wall of a microbial cell by producing a fusion protein. First priority date 08.07.92; published 20.01.94

EP patent application 93201239.6 (not yet published) Production of antibodies or (functionalized) fragments thereof derived from heavy chain immunoglobulins of

15 *Camelidae*. Filing date 29.04.93

Non-patent literature references:

R.E. Bird & B. Webb Walker Single chain antibody variable regions. *TIBTECH* 9 (April 1991) 132-137

20 A. Conzelmann, C. Fankhauser & C. Desponds Myoinositol gets incorporated into numerous membrane glycoproteins of *Saccharomyces cerevisiae*; incorporation is dependent on phosphomannomutase (*SEC53*). *The EMBO Journal* 9, No.3 (1990) 653-661

L. Dente, G. Cesareni & R. Cortese pEMBL: a new family of single stranded

25 plasmids. *Nucleic Acids Research* 11, No. 6 (1983) 1645-1655

E. Erhart, & C.P. Hollenberg The Presence of a Defective *Leu2* Gene on 2 $\mu$  DNA Recombinant Plasmids of *Saccharomyces cerevisiae* Is Responsible for Curing and High Copy Number. *Journal of Bacteriology*. 156, No.2 (Nov. 1983) 625-635

C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E.

30 Bajyana Songa, N. Bendahman & R. Hamers Naturally occurring antibodies devoid of light chains. *Nature* 363 (3 June 1993) 446-448.

M.M. Harmsen, A.C. Langedijk, E. van Tuinen, R.H. Geerse, H.A. Raué, & J. Maat  
Effect of pmr1 disruption and different signal sequences on the intracellular  
processing and secretion of *Cyamopsis tetragonoloba*  $\alpha$ -galactosidase by  
*Saccharomyces cerevisiae*. *Gene* **125** (1993) 115-123

5 T. Ishizaki, N. Hirayama, H. Shinkawa, O. Nimi, & Y. Murooka Nucleotide  
Sequence of the Gene for Cholesterol Oxidase from a *Streptomyces* sp. *Journal of*  
*Bacteriology* **171**, No.1 (Jan. 1989) 596-601

J. Kok, K.J. Leenhouts, A.J. Haandrikman, A.M. Ledeboer & G. Venema Nucleotide  
Sequence of the Cell Wall Proteinase Gene of *Streptococcus cremoris* Wg2. *Applied*  
10 and Environmental Microbiology **54**, No. 1 (Jan. 1988) 231-238

J. Kok Genetics of the proteolytic system of lactic acid bacteria. *FEMS*  
*Microbiology Reviews*. **87** (1990) 15-42, esp. item 3.2 and Figure 7 on pages 22-23

R. Lewin The universal constructor set. *New Scientist* (8 December 1990) 30-33

P.N. Lipke, D. Wojciechowicz & J. Kurjan *AGα1* Is the Structural Gene for the  
15 *Saccharomyces cerevisiae*  $\alpha$ -Agglutinin, a Cell Surface Glycoprotein Involved in Cell-  
Cell Interactions during Mating. *Molecular and Cellular Biology*. **9**, No.8 (Aug.  
1989) 3155-3165.

D.A. Mead, E. Szczesna-Skorupa & B. Kemper Single-stranded DNA 'blue' T7  
promoter plasmids: a versatile tandem promoter system for cloning and protein  
20 engineering. *Protein Engineering* **1**, No. 1 (1986) 67-74

R. Orlandi, D.H. Güssow, P.T. Jones, & G. Winter Cloning immunoglobulin variable  
domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*  
86 (May 1989) 3833-3837

B.C. Ossendorp The non-specific lipid-transfer binding protein: a recombinant DNA  
25 and immunological approach. Thesis University of Utrecht (30 September 1992)

T. Ohta, K. Fujishiro, K. Yamaguchi, Y. Tamura, K. Aisaka, T. Uwajima & M.  
Hasegawa Sequence of gene *choB* encoding cholesterol oxidase of *Brevibacterium*  
*sterolicum*: comparison with *choA* of *Streptomyces* sp. SA-COO. *Gene* **103** (1991)  
93-96

A. Roy, C.F. Lu, D.L. Marykwas, P.N. Lipke & J. Kurjan The *AGA1* Product Is Involved in Cell Surface Attachment of the *Saccharomyces cerevisiae* Cell Adhesion Glycoprotein a-Agglutinin. *Molecular and Cellular Biology* 11 No.8 (Aug. 1991) 4196-4206

5 M.P. Schreuder, S. Brekelmans, H. van den Ende, & F.M. Klis Targeting of a Heterologous Protein to the Cell Wall of *Saccharomyces cerevisiae*. *Yeast* 9 (1993) 399-409

A. Ullrich, L. Coussens, J.S. Hayflick, T.J. Dull, A. Gray, A.W. Tam, J. Lee, Y. Yarden, T.A. Libermann, J. Schlessinger, J. Downward, E.L.V. Mayes, N. Whittle, 10 M.D. Waterfield & P.H. Seuberg Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309 (31 May 1984) 418-425

J.M.A. Verbakel Heterologous gene expression in the yeast *Saccharomyces cerevisiae*. Thesis University of Utrecht (1 May 1991), esp. pages 76-89

15 A. Vrielink, L.F. Lloyd, & D.M. Blow Crystal Structure of Cholesterol Oxidase from *Brevibacterium sterolicum* Refined at 1.8 Å Resolution. *J. Mol. Biol.*, 219 (1991) 533-554

E.S. Ward, D. Güssow, A.D. Griffiths, P.T. Jones & G. Winter. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *E. coli*. 20 *Nature* 341 (12 October 1989) 544-546

25

Information on a deposit of a micro-organism under the Budapest Treaty is given on page 26, lines 5-7 above. In agreement with Rule 28 (4) EPC, or a similar arrangement for a State not being a Contracting State of the EPC, it is hereby requested that a sample of such deposit, when requested, will be submitted to an 30 expert only.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Unilever N.V.  
(B) STREET: Weena 455  
(C) CITY: Rotterdam  
(E) COUNTRY: The Netherlands  
(F) POSTAL CODE (ZIP): NL-3013 AL

(A) NAME: Unilever PLC  
(B) STREET: Unilever House Blackfriars  
(C) CITY: London  
(E) COUNTRY: United Kingdom  
(F) POSTAL CODE (ZIP): EC4P 4BQ

(A) NAME: Leon Gerardus J. FRENKEN  
(B) STREET: Geldersestraat 90  
(C) CITY: Rotterdam  
(E) COUNTRY: The Netherlands  
(F) POSTAL CODE (ZIP): NL-3011 MP

(A) NAME: Pieter DE GEUS  
(B) STREET: Boeier 24  
(C) CITY: Barendrecht  
(E) COUNTRY: The Netherlands  
(F) POSTAL CODE (ZIP): NL-2991 KB

(A) NAME: Franciscus Maria KLIS  
(B) STREET: Benedenlangs 102  
(C) CITY: Amsterdam  
(E) COUNTRY: The Netherlands  
(F) POSTAL CODE (ZIP): NL-1025 KL

(A) NAME: Holger York TOSCHKA; c/o Langnese Iglo, BR3  
(B) STREET: Aeckern 1  
(C) CITY: REKEN  
(E) COUNTRY: Germany  
(F) POSTAL CODE (ZIP): D-48734

(A) NAME: Cornelis Theodorus VERRIPS  
(B) STREET: Hagedoorn 18  
(C) CITY: Maassluis  
(E) COUNTRY: The Netherlands  
(F) POSTAL CODE (ZIP): NL-3142 KB

(ii) TITLE OF INVENTION: Immobilized proteins with specific binding capacities and their use in processes and products.

(iii) NUMBER OF SEQUENCES: 40

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 231 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (vii) IMMEDIATE SOURCE:

(B) CLONE: fragment in pUR4119

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTGGAGC TCATCACACA AACAAACAAA ACAAAATGAT GCTTTGCAA GCCTTCTTT	60
TCCTTTGGC TGGTTTGCA GCCAAAATAT CTGCGCAGGT GCAGCTGCAG TAATGAACCA	120
CGGTCAACCGT CTCCTCAGGT GGAGGCGGTT CAGGCGGAGG TGGCTCTGGC GGTGGCGGAT	180
CGGACATCGA GCTCACTCAG ACCAAGCTCG AGATCAAAACG GTGATAAGCT T	231

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (vii) IMMEDIATE SOURCE:

(B) CLONE: linker XhoI-NheI coding strand

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCGAGATCAA AGGCGGATCT G	21
-------------------------	----

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (vii) IMMEDIATE SOURCE:

(B) CLONE: linker XhoI-NheI non-coding strand

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTAGCAGATC CGCCTTGAT C	21
------------------------	----

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (vii) IMMEDIATE SOURCE:

(B) CLONE: linker EagI-PstI coding strand

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGCCGCCAG GTGCAGCTGC A	21
------------------------	----

## (2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 13 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: linker EagI-PstI non-coding strand

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCTGCACCTG GGC

13

## (2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: PCR primer A (heavy chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGGTSMARCT GCAGSAGTCW GG

22

## (2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: PCR primer B (heavy chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CC

32

## (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: PCR primer C (light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GACATTGAGC TCACCCAGTC TCCA

24

## (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: PCR primer D (light chain)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTGGATCTC GAGCTGGTC CC

22

## (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: linker EcoRI-PstI coding strand

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AATTGGGCCG TTCAGGTGCA GCTGCA

26

## (2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: linker EcoRI-PstI non-coding strand

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCTGCACCTG AACGGCCG

18

## (2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 714 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: ScFv antitraseolide 02/01/01

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CTGCAGGAGT	CTGGACCTGG	CCTGGTAAAA	CCTTCTCAGT	CTCTGTCCCT	CACCTGCACT	60
GTCACTGGCT	ACTCAATCAC	CAGTGATTTC	GCCTGGAAC	GGATCCGGCA	GTTTCCAGGA	120
AACCAACTGG	AGTGGATGGG	CTACATAAGC	TACACTGGTA	GCACTAGCTA	CAACCCATCT	180
CTCAAAAGTC	GAATCTCTCT	CACTCGAGAC	ACATCCAAGA	ACCGAGTTCTT	CCTGCAGTTG	240
AATTCTGTGA	CTACTGAGGA	CACAGCCACA	TATTACTGTG	CAACGTCCCT	AACATGGTTA	300
CTACGTCGGA	AACGTTCTTA	CTGGGGCCAA	GGGACCACGG	TCACCGTCTC	CTCAGGTTGGA	360
GGCGGTTTCAG	GCGGAGGTGG	CTCTGGCGGT	GGCGGATCGG	ACATCGAGCT	CACCCAGTCT	420
CCATCCTCCA	TGTCTGTATC	TCTGGGAGAC	ACAGTCAGCA	TCACTTGCCA	TGCAAGTCAG	480
GACATTAGCA	GTAATATAGG	GTGGTTGCAG	CAGAAACCAAG	GGAAATCATT	TAAGGGCCTG	540
ATCTATCATG	GAACCAACTT	GGAAAGATGGT	ATTCCATCAA	GGTTCAAGTGG	CAGTGGATCT	600
GGAGCAGATT	ATTCCCTCAC	CATCAGCAGC	CTGGAATCTG	AAGATTTGCA	AGACTATTAC	660
TGTGTACAGT	ATGCTCAGTT	TCCATTACAG	TTCCGGCTCGG	GGACCAAGCT	CGAG	714

## (2) INFORMATION FOR SEQ ID NO: 13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 734 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ScFv anti-HCG

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGGCCGTTCA	GGTGCAGCTG	CAGGAGTCTG	GGGGACACTT	AGTGAAGCCT	GGAGGGTCCC	60
TGAAACTCTC	CTGTGCAGCC	TCTGGATTG	CTTTCAGTAG	CTTTGACATG	TCTTGGATTG	120
GCCAGACTCC	GGAGAAGAGG	CTGGAGTGGG	TCGCAAGCAT	TACTAATGTT	GGTACTTTACA	180
CCTACTATCC	AGGCAGTGTG	AAGGGCCGAT	TCTCCATCTC	CAGAGACAAT	GCCAGGAACA	240
CCCTAAACCT	GCAAATGAGC	AGTCTGAGGT	CTGAGGACAC	GGCCTTGTAT	TTCTGTGCAA	300
GACAGGGGAC	TGCGGCACAA	CCTTACTGGT	ACTTGATGT	CTGGGGCCAA	GGGACCACGG	360
TCACCGTCTC	CTCAGGTGGA	GGCGGTTCA	GGGGAGGTGG	CTCTGGCGGT	GGCGGATCGG	420
ACATCGAGCT	CACCCAGTCT	CCAAAATCCA	TGTCCATGTC	CGTAGGAGAG	AGGGTCACCT	480
TGAGCTGCAA	GGCCAGTGAG	ACTGTGGATT	CTTTTGTGTC	CTGGTATCAA	CAGAAACCAAG	540
AACAGTCFCC	TAAATTGTTG	ATATTGGGG	CATCCAACCG	GTTCAGTGGG	GTCCCCGATC	600
GCTTCACTGG	CAGTGGATCT	GCAACAGACT	TCACTCTGAC	CATCAGCAGT	GTGCAGGCTG	660
AGGACTTTGC	GGATTACCAAC	TGTGGACAGA	CTTACAATCA	TCCGTATACG	TTCCGGAGGGG	720
GGACCAAGCT	CGAG					734

## (2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2685 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: *Saccharomyces cerevisiae*
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: pYY105
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 1..2685
  - (D) OTHER INFORMATION: /product= "Flocculation protein"  
/gene= "FLO1"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATG ACA ATG CCT CAT CGC TAT ATG TTT TTG GCA GTC TTT ACA CTT CTG	48
Met Thr Met Pro His Arg Tyr Met Phe Leu Ala Val Phe Thr Leu Leu	
1 5 10 15	
GCA CTA ACT AGT GTG GCC TCA GGA GCC ACA GAG GCG TGC TTA CCA GCA	96
Ala Leu Thr Ser Val Ala Ser Gly Ala Thr Glu Ala Cys Leu Pro Ala	
20 25 30	
GGC CAG AGG AAA ACT GGG ATG AAT ATA AAT TTT TAC CAG TAT TCA TTG	144
Gly Gln Arg Lys Ser Gly Met Asn Ile Asn Phe Tyr Gln Tyr Ser Leu	
35 40 45	
AAA GAT TCC TCC ACA TAT TCG AAT GCA GCA TAT ATG GCT TAT GGA TAT	192
Lys Asp Ser Ser Thr Tyr Ser Asn Ala Ala Tyr Met Ala Tyr Gly Tyr	
50 55 60	
GCC TCA AAA ACC AAA CTA GGT TCT GTC GGA GGA CAA ACT GAT ATC TCG	240
Ala Ser Lys Thr Lys Leu Gly Ser Val Gly Gln Thr Asp Ile Ser	
65 70 75 80	
ATT GAT TAT AAT ATT CCC TGT GTT AGT TCA TCA GGC ACA TTT CCT TGT	288
Ile Asp Tyr Asn Ile Pro Cys Val Ser Ser Gly Thr Phe Pro Cys	
85 90 95	
CCT CAA GAA GAT TCC TAT GGA AAC TGG GGA TGC AAA GGA ATG GGT CCT	336
Pro Gln Glu Asp Ser Tyr Gly Asn Trp Gly Cys Lys Gly Met Gly Ala	
100 105 110	
TGT TCT AAT AGT CAA GGA ATT GCA TAC TGG AGT ACT GAT TTA TTT GGT	384
Cys Ser Asn Ser Gln Gly Ile Ala Tyr Trp Ser Thr Asp Leu Phe Gly	
115 120 125	
TTC TAT ACT ACC CCA ACA AAC GTC ACC CTA GAA ATG ACA GGT TAT TTT	432
Phe Tyr Thr Pro Thr Asn Val Thr Leu Glu Met Thr Gly Tyr Phe	
130 135 140	
TTA CCA CCA CAG ACG GGT TCT TAC ACA TTC AAG TTT GCT ACA GTT GAC	480
Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys Phe Ala Thr Val Asp	
145 150 155 160	
GAC TCT GCA ATT CTA TCA GTC GGT GGT GCA ACC GCG TTC AAC TGT TGT	528
Asp Ser Ala Ile Leu Ser Val Gly Gly Ala Thr Ala Phe Asn Cys Cys	
165 170 175	

GCT CAA CAG CAA CCG CCG ATC ACA TCA ACG AAC TTT ACC ATT GAC GGT Ala Gln Gln Gln Pro Pro Ile Thr Ser Thr Asn Phe Thr Ile Asp Gly 180 185 190	576
ATC AAG CCA TGG GGT GGA AGT TTG CCA CCT AAT ATC GAA GGA ACC GTC Ile Lys Pro Trp Gly Gly Ser Leu Pro Pro Asn Ile Glu Gly Thr Val 195 200 205	624
TAT ATG TAC GCT GGC TAC TAT TAT CCA ATG AAG GTT GTT TAC TCG AAC Tyr Met Tyr Ala Gly Tyr Tyr Pro Met Lys Val Val Tyr Ser Asn 210 215 220	672
GCT GTT TCT TGG GGT ACA CTT CCA ATT AGT GTG ACA CTT CCA GAT GGT Ala Val Ser Trp Gly Thr Leu Pro Ile Ser Val Thr Leu Pro Asp Gly 225 230 235 240	720
ACC ACT GTA AGT GAT GAC TTC GAA GGG TAC GTC TAT TCC TTT GAC GAT Thr Thr Val Ser Asp Asp Phe Glu Gly Tyr Val Tyr Ser Phe Asp Asp 245 250 255	768
GAC CTA AGT CAA TCT AAC TGT ACT GTC CCT GAC CCT TCA AAT TAT GCT Asp Leu Ser Gln Ser Asn Cys Thr Val Pro Asp Pro Ser Asn Tyr Ala 260 265 270	816
GTC AGT ACC ACT ACA ACT ACA ACG GAA CCA TGG ACC GGT ACT TTC ACT Val Ser Thr Thr Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr 275 280 285	864
TCT ACA TCT ACT GAA ATG ACC ACC GTC ACC GGT ACC AAC GGC GTT CCA Ser Thr Ser Thr Glu Met Thr Thr Val Thr Gly Thr Asn Gly Val Pro 290 295 300	912
ACT GAC GAA ACC GTC ATT GTC ATC AGA ACT CCA ACC AGT GAA GGT CTA Thr Asp Glu Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu 305 310 315 320	960
ATC AGC ACC ACC ACT GAA CCA TGG ACT GGC ACT TTC ACT TCG ACT TCC Ile Ser Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser 325 330 335	1008
ACT GAG GTT ACC ACC ATC ACT GGA ACC AAC GGT CAA CCA ACT GAC GAA Thr Glu Val Thr Thr Ile Thr Gly Thr Asn Gly Gln Pro Thr Asp Glu 340 345 350	1056
ACT GTG ATT GTT ATC AGA ACT CCA ACC AGT GAA GGT CTA ATC AGC ACC Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu Ile Ser Thr 355 360 365	1104
ACC ACT GAA CCA TGG ACT GGT ACT TTC ACT TCT ACA TCT ACT GAA ATG Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met 370 375 380	1152
ACC ACC GTC ACC GGT ACT AAC GGT CAA CCA ACT GAC GAA ACC GTG ATT Thr Thr Val Thr Gly Thr Asn Gly Gln Pro Thr Asp Glu Thr Val Ile 385 390 395 400	1200
GTT ATC AGA ACT CCA ACC AGT GAA GGT TTG GTT ACA ACC ACC ACT GAA Val Ile Arg Thr Pro Thr Ser Glu Gly Leu Val Thr Thr Thr Glu 405 410 415	1248
CCA TGG ACT GGT ACT TTT ACT TCG ACT TCC ACT GAA ATG TCT ACT GTC Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met Ser Thr Val 420 425 430	1296
ACT GGA ACC AAT GGC TTG CCA ACT GAT GAA ACT GTC ATT GTT GTC AAA Thr Gly Thr Asn Gly Leu Pro Thr Asp Glu Thr Val Ile Val Val Lys 435 440 445	1344

ACT CCA ACT ACT GCC ATC TCA TCC AGT TTG TCA TCA TCA TCT TCA GGA Thr Pro Thr Thr Ala Ile Ser Ser Ser Leu Ser Ser Ser Ser Ser Gly 450 455 460	1392
CAA ATC ACC AGC TCT ATC ACG TCT TCG CGT CCA ATT ATT ACC CCA TTC Gln Ile Thr Ser Ser Ile Thr Ser Ser Arg Pro Ile Ile Thr Pro Phe 465 470 475 480	1440
TAT CCT AGC AAT GGA ACT TCT GTG ATT TCT TCC TCA GTA ATT TCT TCC Tyr Pro Ser Asn Gly Thr Ser Val Ile Ser Ser Ser Val Ile Ser Ser 485 490 495	1488
TCA GTC ACT TCT TCT CTA TTC ACT TCT TCT CCA GTC ATT TCT TCC TCA Ser Val Thr Ser Ser Leu Phe Thr Ser Ser Pro Val Ile Ser Ser Ser 500 505 510	1536
GTC ATT TCT TCT TCT ACA ACA ACC TCC ACT TCT ATA TTT TCT GAA TCA Val Ile Ser Ser Ser Thr Thr Ser Thr Ser Ile Phe Ser Glu Ser 515 520 525	1584
TCT AAA TCA TCC GTC ATT CCA ACC AGT AGT TCC ACC TCT GGT TCT TCT Ser Lys Ser Ser Val Ile Pro Thr Ser Ser Ser Thr Ser Gly Ser Ser 530 535 540	1632
GAG AGC GAA AGC AGT TCA GCT GGT TCT GTC TCT TCT TCC TCT TCT TTT ATC Glu Ser Glu Thr Ser Ser Ala Gly Ser Val Ser Ser Ser Phe Ile 545 550 555 560	1680
TCT TCT GAA TCA TCA AAA TCT CCT ACA TAT TCT TCT TCA TCA TTA CCA Ser Ser Glu Ser Ser Lys Ser Pro Thr Tyr Ser Ser Ser Ser Leu Pro 565 570 575	1728
CTT GTT ACC AGT GCG ACA ACA AGC CAG GAA ACT GCT TCT TCA TCA TTA CCA Leu Val Thr Ser Ala Thr Ser Gln Glu Thr Ala Ser Ser Leu Pro 580 585 590	1776
CCT GCT ACC ACT ACA AAA ACG AGC GAA CAA ACC ACT TTG GTT ACC GTG Pro Ala Thr Thr Lys Thr Ser Glu Gln Thr Thr Leu Val Thr Val 595 600 605	1824
ACA TCC TGC GAG TCT CAT GTG TGC ACT GAA TCC ATC TCC CCT GCG ATT Thr Ser Cys Glu Ser His Val Cys Thr Glu Ser Ile Ser Pro Ala Ile 610 615 620	1872
GTT TCC ACA GCT ACT GTT ACT GTT AGC GGC GTC ACA ACA GAG TAT ACC Val Ser Thr Ala Thr Val Thr Val Ser Gly Val Thr Thr Glu Tyr Thr 625 630 635 640	1920
ACA TGG TGC CCT ATT TCT ACT ACA GAG ACA ACA AAG CAA ACC AAA GGG Thr Trp Cys Pro Ile Ser Thr Thr Glu Thr Thr Lys Gln Thr Lys Gly 645 650 655	1968
ACA ACA GAG CAA ACC ACA GAA ACA ACA AAA CAA ACC ACG GTA GTT ACA Thr Thr Glu Gln Thr Thr Glu Thr Thr Lys Gln Thr Thr Val Val Thr 660 665 670	2016
ATT TCT TCT TGT GAA TCT GAC GTA TGC TCT AAG ACT GCT TCT CCA GCC Ile Ser Ser Cys Glu Ser Asp Val Cys Ser Lys Thr Ala Ser Pro Ala 675 680 685	2064
ATT GTA TCT ACA AGC ACT GCT ACT ATT AAC GGC GTT ACT ACA GAA TAC Ile Val Ser Thr Ser Thr Ala Thr Ile Asn Gly Val Thr Thr Glu Tyr 690 695 700	2112
ACA ACA TGG TGT CCT ATT TCC ACC ACA GAA TCG AGG CAA CAA ACA ACG Thr Thr Trp Cys Pro Ile Ser Thr Thr Glu Ser Arg Gln Gln Thr Thr 705 710 715 720	2160

CTA GTT ACT GTT ACT TCC TGC GAA TCT GGT GTG TGT TCC GAA ACT GCT Leu Val Thr Val Thr Ser Cys Glu Ser Gly Val Cys Ser Glu Thr Ala 725 730 735	2208
TCA CCT GCC ATT GTT TCG ACG GCC ACG GCT ACT GTG AAT GAT GTT GTT Ser Pro Ala Ile Val Ser Thr Ala Thr Val Asn Asp Val Val 740 745 750	2256
ACG GTC TAT CCT ACA TGG AGG CCA CAG ACT GCG AAT GAA GAG TCT GTC Thr Val Tyr Pro Thr Trp Arg Pro Gln Thr Ala Asn Glu Glu Ser Val 755 760 765	2304
AGC TCT AAA ATG AAC AGT GCT ACC GGT GAG ACA ACA ACC AAT ACT TTA Ser Ser Lys Met Asn Ser Ala Thr Gly Glu Thr Thr Asn Thr Leu 770 775 780	2352
GCT GCT GAA ACG ACT ACC AAT ACT GTC GCT GCT GAG ACG ATT ACC AAT Ala Ala Glu Thr Thr Asn Thr Val Ala Ala Glu Thr Ile Thr Asn 785 790 795 800	2400
ACT GGA GCT GCT GAG ACG AAA ACA GTC GTC ACC TCT TCG CTT TCA AGA Thr Gly Ala Ala Glu Thr Lys Thr Val Val Thr Ser Ser Leu Ser Arg 805 810 815	2448
TCT AAT CAC GCT GAA ACA CAG ACG GCT TCC GCG ACC GAT GTG ATT GGT Ser Asn His Ala Glu Thr Gln Thr Ala Ser Ala Thr Asp Val Ile Gly 820 825 830	2496
CAC AGC AGT AGT GTT GTT TCT GTC TCC GAA ACT GGC AAC ACC AAG AGT His Ser Ser Val Val Ser Val Ser Glu Thr Gly Asn Thr Lys Ser 835 840 845	2544
CTA ACA AGT TCC GGG TTG AGT ACT ATG TCG CAA CAG CCT CGT AGC ACA Leu Thr Ser Ser Gly Leu Ser Thr Met Ser Gln Gln Pro Arg Ser Thr 850 855 860	2592
CCA GCA AGC AGC ATG GTC GGA TAT AGT ACA GCT TCT TTA GAA ATT TCA Pro Ala Ser Ser Met Val Gly Tyr Ser Thr Ala Ser Leu Glu Ile Ser 865 870 875 880	2640
ACG TAT GCT GGC AGT GCA ACA GCT TAC TGG CCG GTC GTG GTT TAA Thr Tyr Ala Gly Ser Ala Thr Ala Tyr Trp Pro Val Val Val 885 890 895	2685

## (2) INFORMATION FOR SEQ ID NO: 15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 894 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Met Thr Met Pro His Arg Tyr Met Phe Leu Ala Val Phe Thr Leu Leu 1 5 10 15
Ala Leu Thr Ser Val Ala Ser Gly Ala Thr Glu Ala Cys Leu Pro Ala 20 25 30
Gly Gln Arg Lys Ser Gly Met Asn Ile Asn Phe Tyr Gln Tyr Ser Leu 35 40 45
Lys Asp Ser Ser Thr Tyr Ser Asn Ala Ala Tyr Met Ala Tyr Gly Tyr 50 55 60

Ala Ser Lys Thr Lys Leu Gly Ser Val Gly Gly Gln Thr Asp Ile Ser  
65 70 75 80

Ile Asp Tyr Asn Ile Pro Cys Val Ser Ser Ser Gly Thr Phe Pro Cys  
65 90 95

Pro Gln Glu Asp Ser Tyr Gly Asn Trp Gly Cys Lys Gly Met Gly Ala  
100 105 110

Cys Ser Asn Ser Gln Gly Ile Ala Tyr Trp Ser Thr Asp Leu Phe Gly  
115 120 125

Phe Tyr Thr Thr Pro Thr Asn Val Thr Leu Glu Met Thr Gly Tyr Phe  
130 135 140

Leu Pro Pro Gln Thr Gly Ser Tyr Thr Phe Lys Phe Ala Thr Val Asp  
145 150 155 160

Asp Ser Ala Ile Leu Ser Val Gly Gly Ala Thr Ala Phe Asn Cys Cys  
165 170 175

Ala Gln Gln Gln Pro Pro Ile Thr Ser Thr Asn Phe Thr Ile Asp Gly  
180 185 190

Ile Lys Pro Trp Gly Gly Ser Leu Pro Pro Asn Ile Glu Gly Thr Val  
195 200 205

Tyr Met Tyr Ala Gly Tyr Tyr Pro Met Lys Val Val Tyr Ser Asn  
210 215 220

Ala Val Ser Trp Gly Thr Leu Pro Ile Ser Val Thr Leu Pro Asp Gly  
225 230 235 240

Thr Thr Val Ser Asp Asp Phe Glu Gly Tyr Val Tyr Ser Phe Asp Asp  
245 250 255

Asp Leu Ser Gln Ser Asn Cys Thr Val Pro Asp Pro Ser Asn Tyr Ala  
260 265 270

Val Ser Thr Thr Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr  
275 280 285

Ser Thr Ser Thr Glu Met Thr Thr Val Thr Gly Thr Asn Gly Val Pro  
290 295 300

Thr Asp Glu Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu  
305 310 315 320

Ile Ser Thr Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser  
325 330 335

Thr Glu Val Thr Thr Ile Thr Gly Thr Asn Gly Gln Pro Thr Asp Glu  
340 345 350

Thr Val Ile Val Ile Arg Thr Pro Thr Ser Glu Gly Leu Ile Ser Thr  
355 360 365

Thr Thr Glu Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met  
370 375 380

Thr Thr Val Thr Gly Thr Asn Gly Gln Pro Thr Asp Glu Thr Val Ile  
385 390 395 400

Val Ile Arg Thr Pro Thr Ser Glu Gly Leu Val Thr Thr Thr Glu  
405 410 415

Pro Trp Thr Gly Thr Phe Thr Ser Thr Ser Thr Glu Met Ser Thr Val  
420 425 430

Thr Gly Thr Asn Gly Leu Pro Thr Asp Glu Thr Val Ile Val Val Lys  
435 440 445

Thr Pro Thr Thr Ala Ile Ser Ser Ser Leu Ser Ser Ser Ser Gly  
450 455 460

Gln Ile Thr Ser Ser Ile Thr Ser Ser Arg Pro Ile Ile Thr Pro Phe  
465 470 475 480

Tyr Pro Ser Asn Gly Thr Ser Val Ile Ser Ser Ser Val Ile Ser Ser  
485 490 495

Ser Val Thr Ser Ser Leu Phe Thr Ser Ser Pro Val Ile Ser Ser Ser  
500 505 510

Val Ile Ser Ser Ser Thr Thr Ser Thr Ser Ile Phe Ser Glu Ser  
515 520 525

Ser Lys Ser Ser Val Ile Pro Thr Ser Ser Ser Thr Ser Gly Ser Ser  
530 535 540

Glu Ser Glu Thr Ser Ser Ala Gly Ser Val Ser Ser Ser Ser Phe Ile  
545 550 555 560

Ser Ser Glu Ser Ser Lys Ser Pro Thr Tyr Ser Ser Ser Ser Leu Pro  
565 570 575

Leu Val Thr Ser Ala Thr Thr Ser Gln Glu Thr Ala Ser Ser Leu Pro  
580 585 590

Pro Ala Thr Thr Thr Lys Thr Ser Glu Gln Thr Thr Leu Val Thr Val  
595 600 605

Thr Ser Cys Glu Ser His Val Cys Thr Glu Ser Ile Ser Pro Ala Ile  
610 615 620

Val Ser Thr Ala Thr Val Thr Val Ser Gly Val Thr Thr Glu Tyr Thr  
625 630 635 640

Thr Trp Cys Pro Ile Ser Thr Thr Glu Thr Thr Lys Gln Thr Lys Gly  
645 650 655

Thr Thr Glu Gln Thr Thr Glu Thr Thr Lys Gln Thr Thr Val Val Thr  
660 665 670

Ile Ser Ser Cys Glu Ser Asp Val Cys Ser Lys Thr Ala Ser Pro Ala  
675 680 685

Ile Val Ser Thr Ser Thr Ala Thr Ile Asn Gly Val Thr Thr Glu Tyr  
690 695 700

Thr Thr Trp Cys Pro Ile Ser Thr Thr Glu Ser Arg Gln Gln Thr Thr  
705 710 715 720

Leu Val Thr Val Thr Ser Cys Glu Ser Gly Val Cys Ser Glu Thr Ala  
725 730 735

Ser Pro Ala Ile Val Ser Thr Ala Thr Ala Thr Val Asn Asp Val Val  
740 745 750

Thr Val Tyr Pro Thr Trp Arg Pro Gln Thr Ala Asn Glu Glu Ser Val  
755 760 765

Ser Ser Lys Met Asn Ser Ala Thr Gly Glu Thr Thr Thr Asn Thr Leu  
 770 775 780

Ala Ala Glu Thr Thr Asn Thr Val Ala Ala Glu Thr Ile Thr Asn  
 785 790 795 800

Thr Gly Ala Ala Glu Thr Lys Thr Val Val Thr Ser Ser Leu Ser Arg  
 805 810 815

Ser Asn His Ala Glu Thr Gln Thr Ala Ser Ala Thr Asp Val Ile Gly  
 820 825 830

His Ser Ser Ser Val Val Ser Val Ser Glu Thr Gly Asn Thr Lys Ser  
 835 840 845

Leu Thr Ser Ser Gly Leu Ser Thr Met Ser Gln Gln Pro Arg Ser Thr  
 850 855 860

Pro Ala Ser Ser Met Val Gly Tyr Ser Thr Ala Ser Leu Glu Ile Ser  
 865 870 875 880

Thr Tyr Ala Gly Ser Ala Thr Ala Tyr Trp Pro Val Val Val  
 885 890

## (2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
 

- (B) CLONE: ChoB template coding strand

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GCCCCCAGCC GCACCCCTCG

19

## (2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
 

- (B) CLONE: ChoB template non-coding strand

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CGAGGGTGCG GCTGGGGGC

19

## (2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: cho01pcr primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

AGATCTGAAT TCGCGGCCGC CCCCCAGCCGC ACCCTCG

37

## (2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: cho02pcr primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AGATCTAACG TTTCAGCTAG CCTGGATGTC GGACGAGATG AT

42

## (2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ChoB template coding strand

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ATCATCTCGT CCGACATCCA G

21

## (2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ChoB template non-coding strand

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CTGGATGTCG GACGAGATGA T

21

## (2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 42 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: mutagenesis primer ChoB

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CGCGGGCGACG GCACCGCCGT ATGCACTGGC GATGACGAGG GC

42

## (2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 42 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: ChoB template coding strand

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCCCTCGTCA TCGGCAGTGG ATACGGCGGT GCCGTCGCCG CG

42

## (2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer prt1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

AAGATCTATC GATCTTGTAA GCCGGTACA

29

## (2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: proteinase template non-coding strand

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GACTGTACCG GCTAACAAAGA TCGATAGCCC TT

32

## (2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: proteinase template coding strand
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTCGGCGAAA TCCAAGCAAA GGCGGCT

27

## (2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: prt2 primer
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CCCAAGCTTC CCCCCGGCCG TTGCTTGGAT TTTCGCCGAC

39

## (2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: EGFl primer
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GGGGCGGCCG CGCTGGAGGA AAAGAAAGTT TGC

33

## (2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: EGF receptor template non-coding strand
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GCAAACCTTTC TTTTCCTCCA GAGCCCGACT CGC

33

## (2) INFORMATION FOR SEQ ID NO: 30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: EGF receptor template coding strand

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

AATGGGCCTA AGATCCCGTC CATGCCACT

30

## (2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: EGF2 primer

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CCCCAAGCTT AAGGCTAGCG GACGGGATCT TAGGCCATT

40

## (2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 177 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: VhC - AGal linker with MycT and Hinge

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GAATTCCAGG TCACCGTCTC CTCAGAACAA AAACTCATCT CAGAAGAGGA TCTGAATGAA

60

CCAAAGATTC CACAACCTCA ACCAAAGCCA CAACCTCAAC CACAACCACA ACCAAAACCT

120

CAACCAAAGC CAGAACCCAGA ATCTACTTCC CCAAAGTCTC CAGCTAGCCT TAAGCTT

177

## (2) INFORMATION FOR SEQ ID NO: 33:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 63 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: VhC - AGal linker with MycT

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GAATTCCAGG TCACCGTCTC CTCAGAACAA AAACATCATCT CAGAAGAGGA TCTGAATGCT	60
AGC	63

## (2) INFORMATION FOR SEQ ID NO: 34:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 144 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: VhC - AGal linker with Hinge

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GAATTCCAGG TCACCGTCTC CTCAGAACCA AAGATTCCAC AACCTCAACC AAAGCCACAA	60
CCTCAACCAC AACCAACCAACC AAAACCTCAA CCAAAGCCAG AACCCAGAATC TACTTCCCCA	120
AAGTCTCCAG CTAGCCTTAA GCTT	144

## (2) INFORMATION FOR SEQ ID NO: 35:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 119 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: fragment in pUR4421 coding strand

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

AATTTAGCGG CCGCCCAGGT GAAACTGCTC GAGTAAGTGA CTAAGGTAC CGTCTCCTCA	60
GAACAAAAAC TCATCTCAGA AGAGGGATCTG AATTAATGAG AATTCACTAA ACGGTGATA	119

## (2) INFORMATION FOR SEQ ID NO: 36:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 119 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: fragment in pUR4421 non-coding strand

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

AGCTTATCAC CGTTTGATGA ATTCTCATTA ATTCAAGATCC TCTTCTGAGA TGAGTTTTTG	60
TTCTGAGGAG ACGGTGACCT TAGTCACTTA CTCGAGCAGT TTCACCTGGG CGGCCGCTA	119

## (2) INFORMATION FOR SEQ ID NO: 37:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 11 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: Myc tail

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn  
1 5 10

## (2) INFORMATION FOR SEQ ID NO: 38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: BstEII-HindIII linker coding strand

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GTCACCGTCT CCTCATAATG A

21

## (2) INFORMATION FOR SEQ ID NO: 39:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: BstEII HindIII linker non-coding strand

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

AGCTTCATTA TGAGGAGACG

20

## (2) INFORMATION FOR SEQ ID NO: 40:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (vii) IMMEDIATE SOURCE: (B) CLONE: primer cho03pcr

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

CGGATCCAAG CTTGAGCCTG GATGTCGGAC GAGATGAT

38

CLAIMS

1. A method for immobilizing a **binding protein** capable of binding to a specific compound, comprising the use of recombinant DNA techniques for producing said binding protein or a functional part thereof still having said specific binding capability, said protein or said part thereof being linked to the outside of a host cell, whereby said binding protein or said part thereof is localized in the cell wall or at the exterior of the cell wall by allowing the host cell to produce and secrete a chimeric protein in which said binding protein or said functional part thereof is bound with its C-terminus to the N-terminus of an anchoring part of an anchoring protein capable of anchoring in the cell wall of the host cell, which anchoring part is derivable from the C-terminal part of said anchoring protein.
2. The method of claim 1, in which the host is selected from the group consisting of Gram-positive bacteria and fungi.
3. The method of claim 2, in which the host is a Gram-positive bacterium selected from the group consisting of lactic acid bacteria, and bacteria belonging to the genera *Bacillus* and *Streptomyces*.
4. The method of claim 2, in which the host is a fungus selected from the group consisting of yeasts belonging to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia* and *Saccharomyces*, and moulds belonging to the genera *Aspergillus*, *Penicillium* and *Rhizopus*.
5. A recombinant polynucleotide comprising
  - (i) a structural gene encoding a binding protein or a functional part thereof still having the specific binding capability, and
  - (ii) at least part of a gene encoding an anchoring protein capable of anchoring in the cell wall of a Gram-positive bacterium or a fungus, said part of a gene encoding at least the anchoring part of said anchoring protein, which

anchoring part is derivable from the C-terminal part of said anchoring protein.

6. The polynucleotide of claim 5, wherein the anchoring protein is selected from the group consisting of  $\alpha$ -agglutinin, a-agglutinin, FLO1, the Major Cell Wall Protein of a fungus, and proteinase of lactic acid bacteria.

7. The polynucleotide of claim 5, further comprising a nucleotide sequence encoding a signal peptide ensuring secretion of the expression product of the polynucleotide.

8. The polynucleotide of claim 7, wherein the signal peptide is derived from a protein selected from the group consisting of the  $\alpha$ -mating factor of yeast,  $\alpha$ -agglutinin of yeast, invertase of *Saccharomyces*, inulinase of *Kluyveromyces*,  $\alpha$ -amylase of *Bacillus*, and proteinase of lactic acid bacteria.

9. The polynucleotide of any of claims 5-8, operably linked to a promoter, which can be an inducible promoter.

10. A recombinant vector comprising a polynucleotide as claimed in any of claims 5-9.

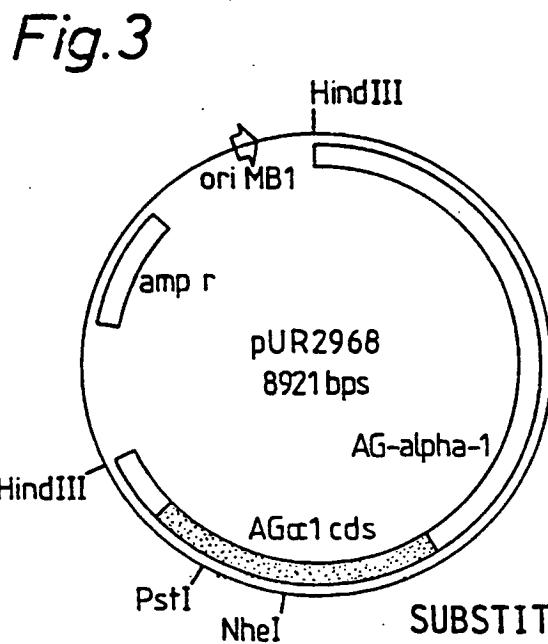
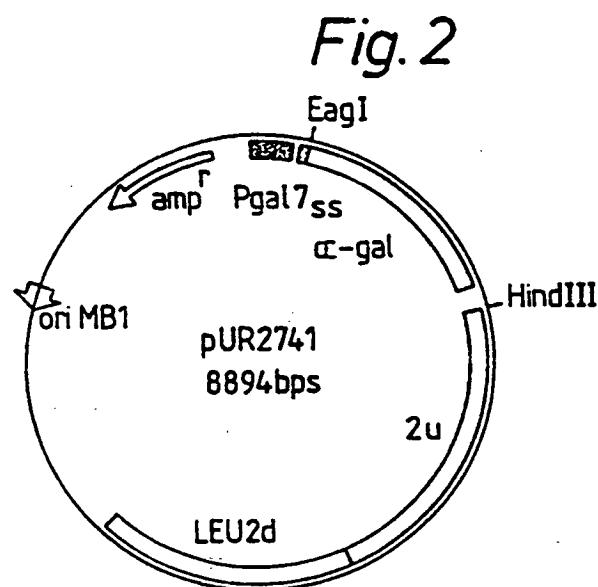
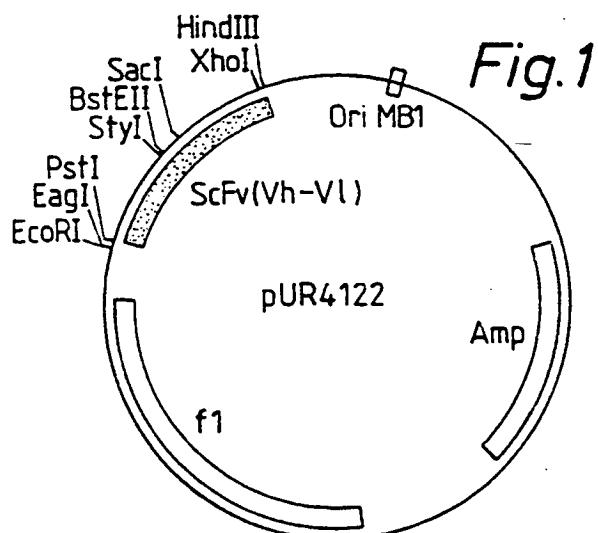
11. A chimeric protein encoded by a polynucleotide as claimed in any of claims 5-9.

12. A host cell having a cell wall at the outside of its cell and containing at least one polynucleotide as claimed in any of claims 5-9.

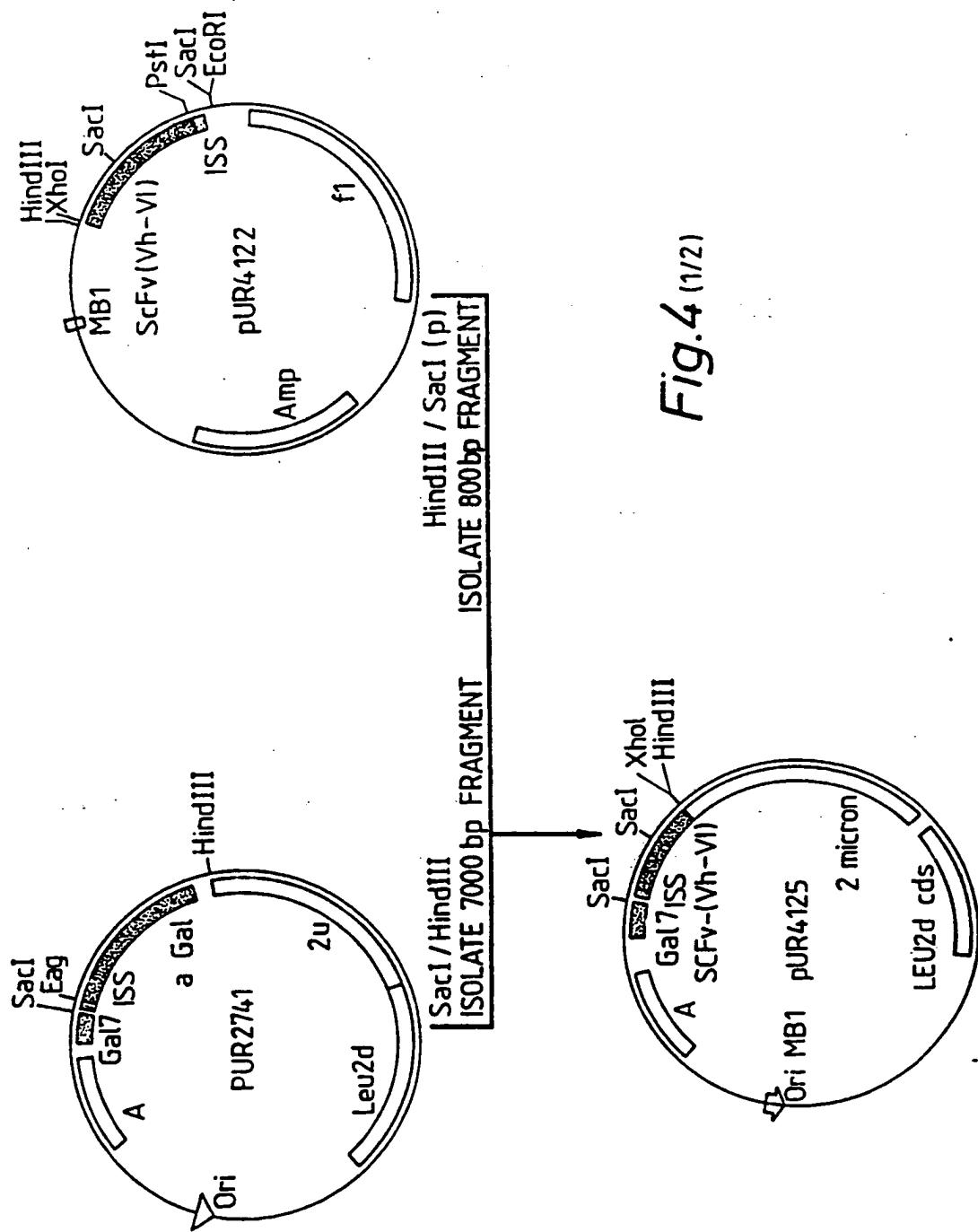
13. The host cell of claim 12, having at least one polynucleotide as claimed in any of claims 5-9 integrated in its chromosome.

14. A host cell having a chimeric protein as claimed in claim 11 immobilized in its cell wall and having the binding protein part of the chimeric protein localized in the cell wall or at the exterior of the cell wall.
15. The host cell of any of claims 12-14, which is a fungus selected from the group consisting of yeasts and moulds.
16. A process for carrying out an isolation process by using an immobilized binding protein or functional part thereof still capable of binding to a specific compound, wherein a medium containing said specific compound is contacted with a host cell as claimed in any of claims 12-15 under conditions whereby a complex between said specific compound and said immobilized binding protein is formed, separating said complex from the medium originally containing said specific compound and, optionally, releasing said specific compound from said binding protein or functional part thereof.

\*\*\*\*\*



SUBSTITUTE SHEET (RULE 26)



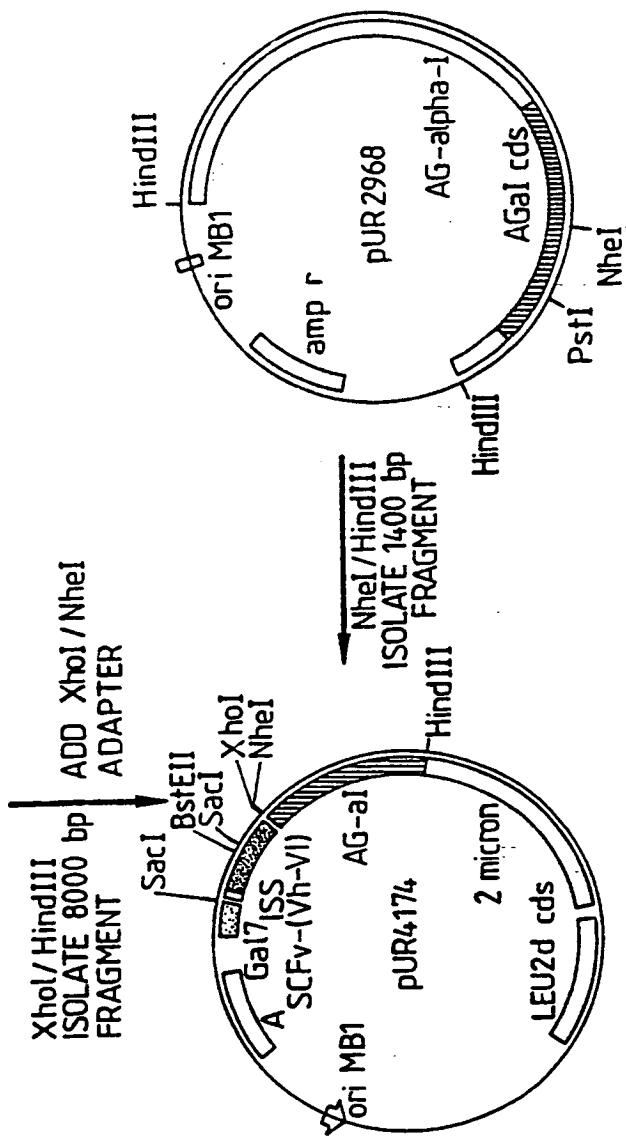


Fig. 4 (1/2) (Cont.)

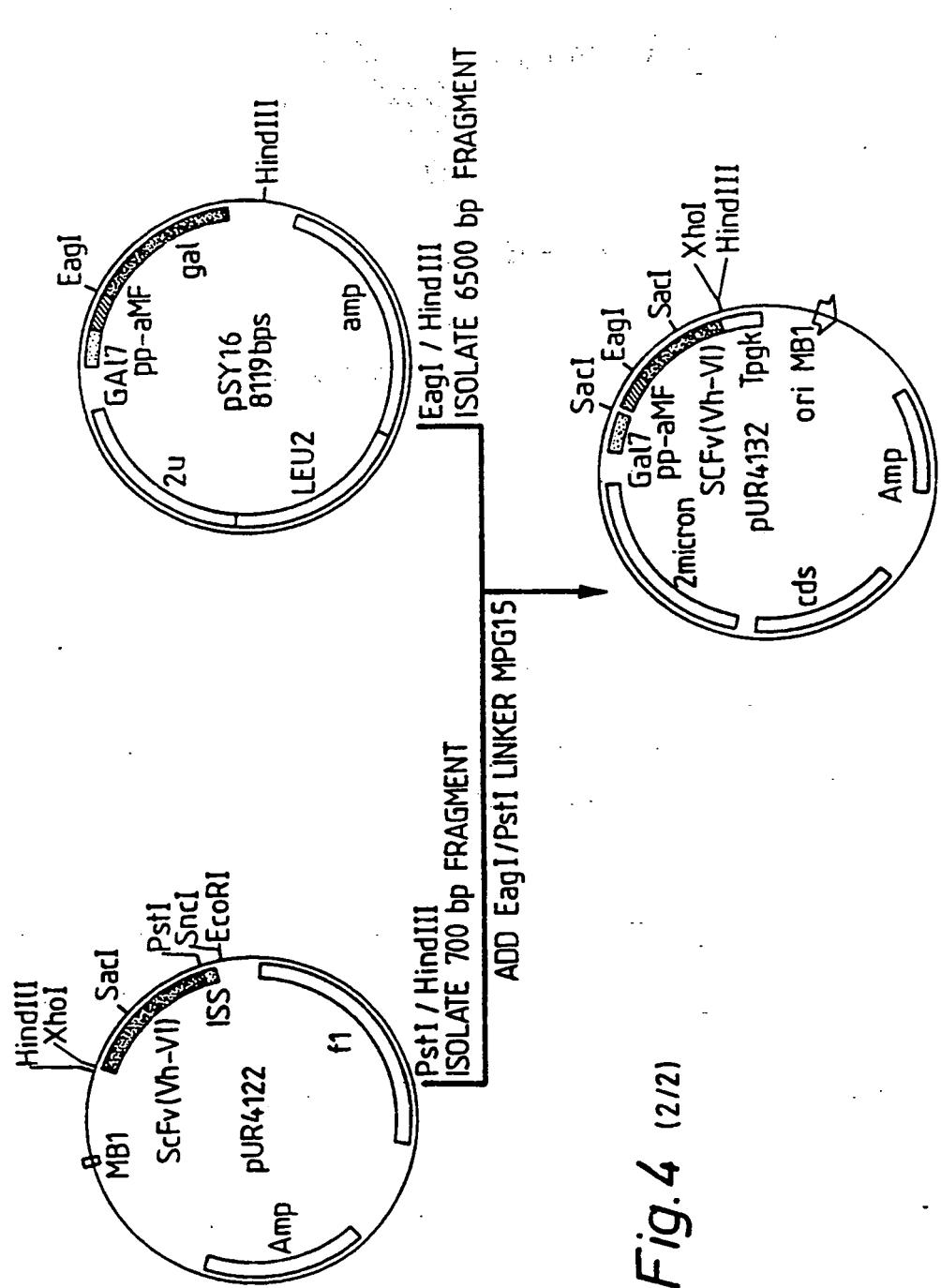


Fig. 4 (2/2)

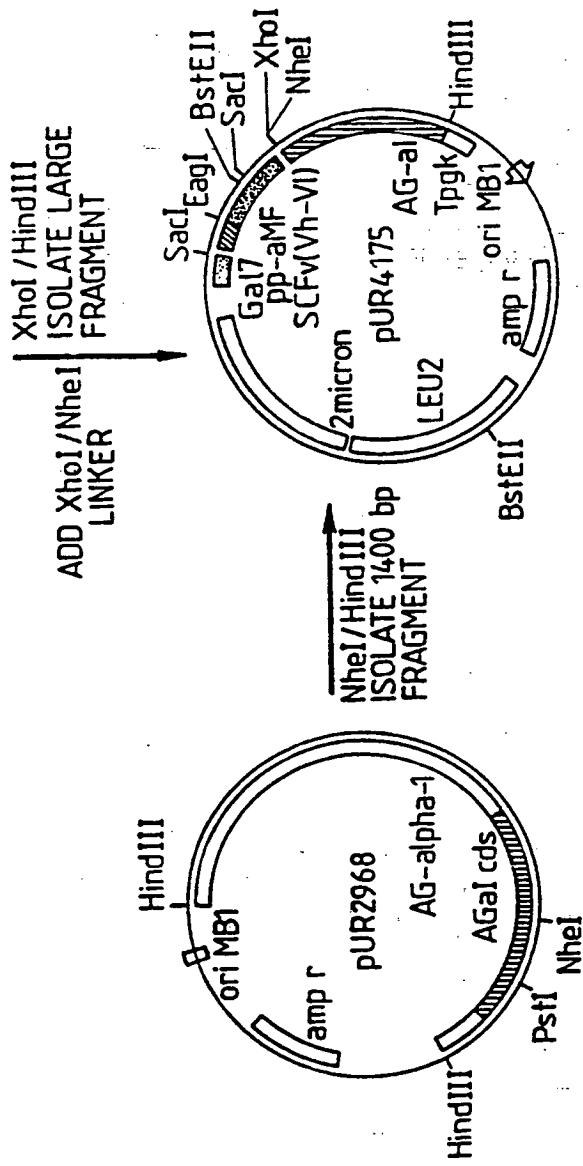


Fig. 4 (2/2) (Cont.)

Fig. 5

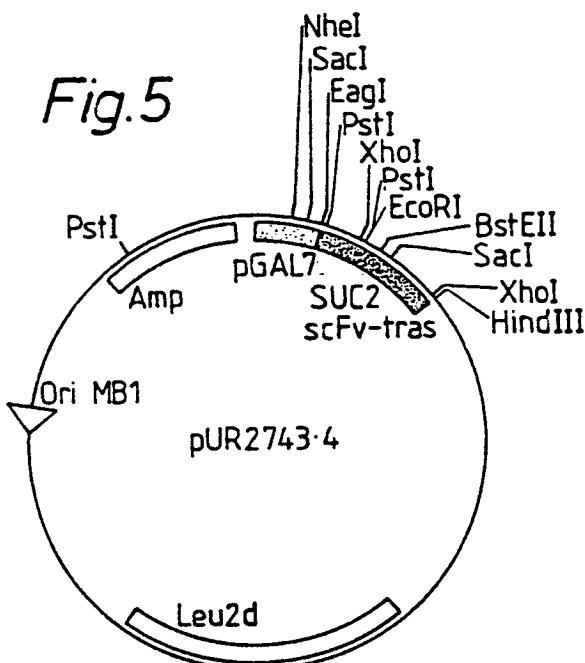


Fig. 6

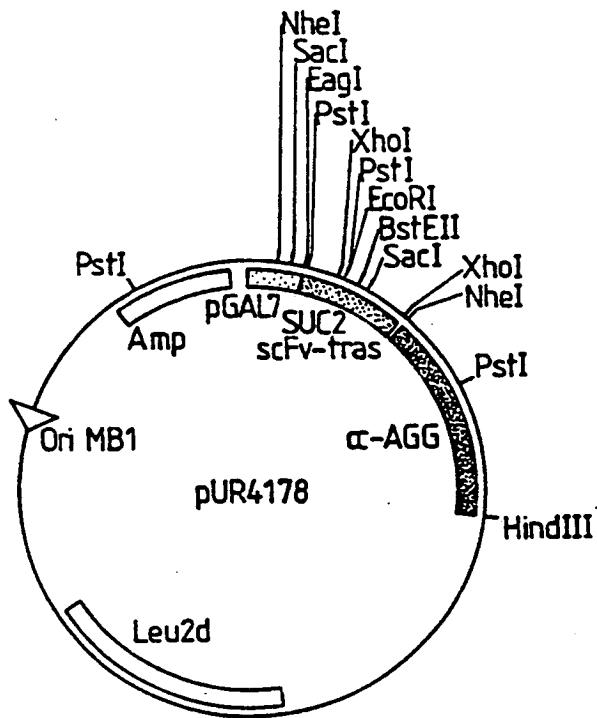
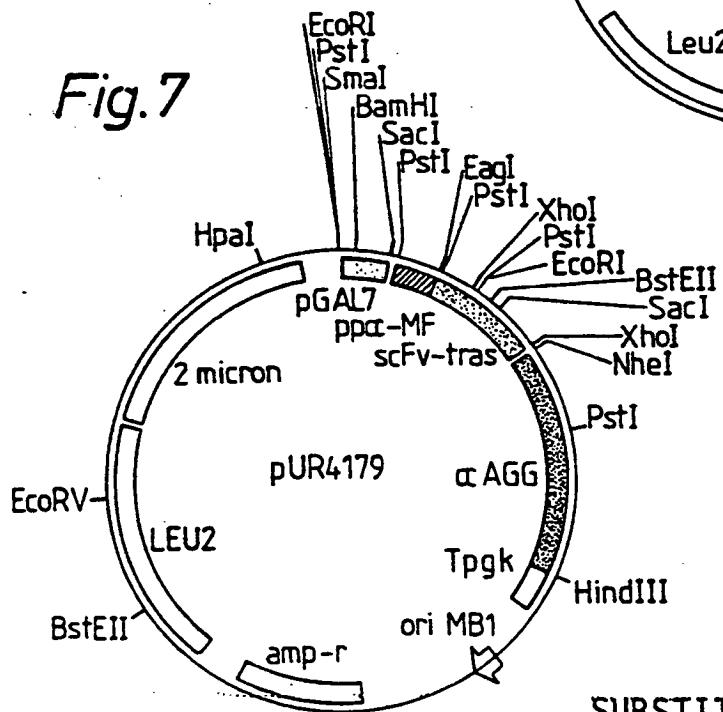
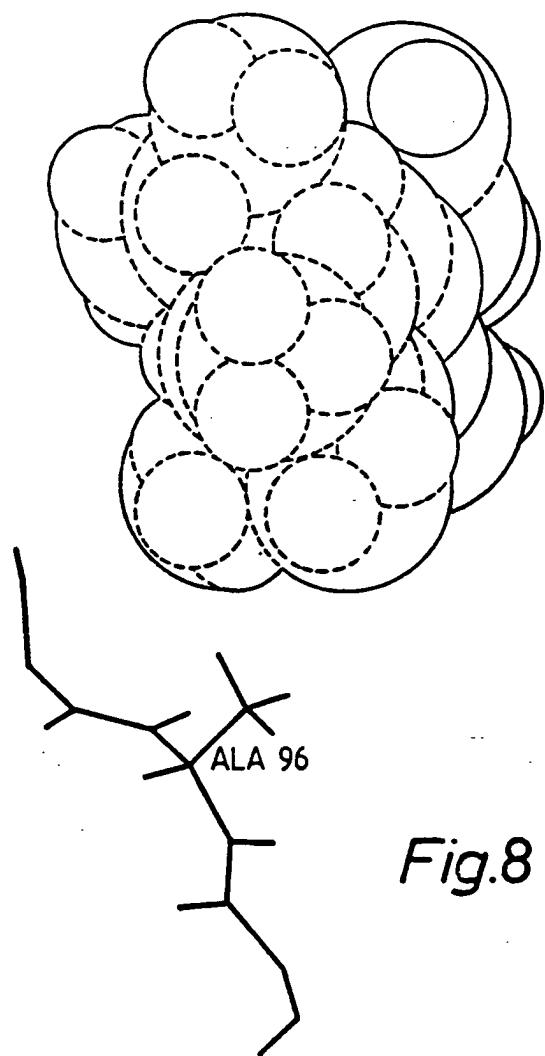


Fig. 7





*Fig.8*

SUBSTITUTE SHEET (RULE 26)

Fig. 9

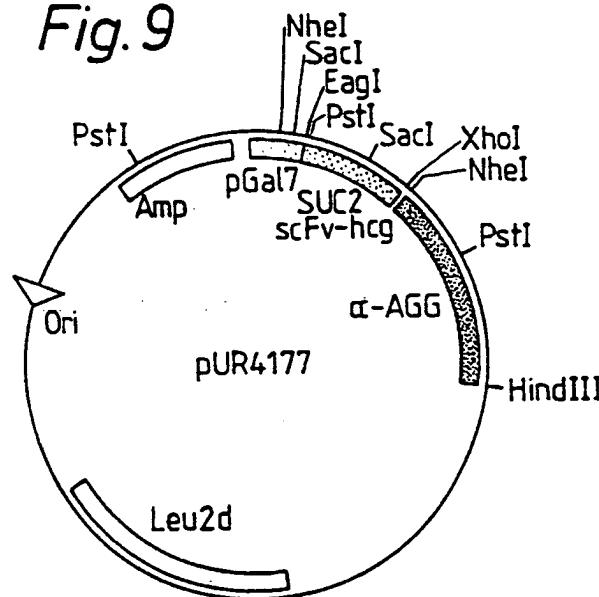


Fig. 10

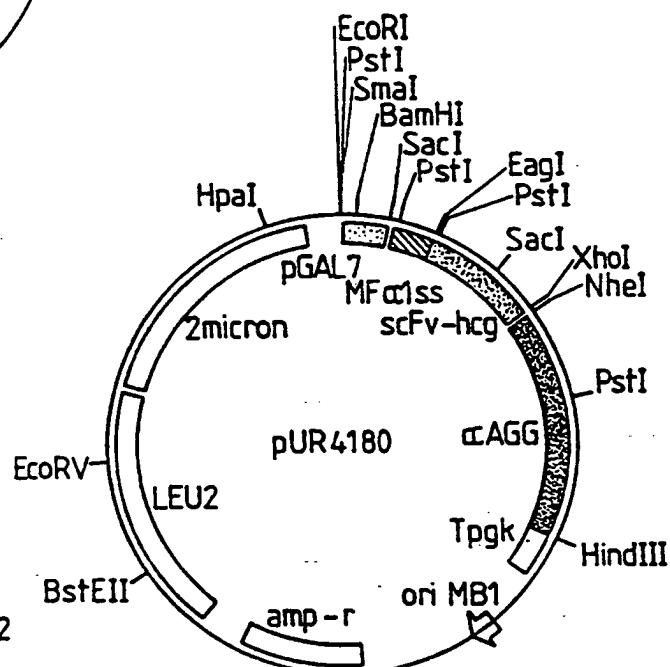


Fig. 11

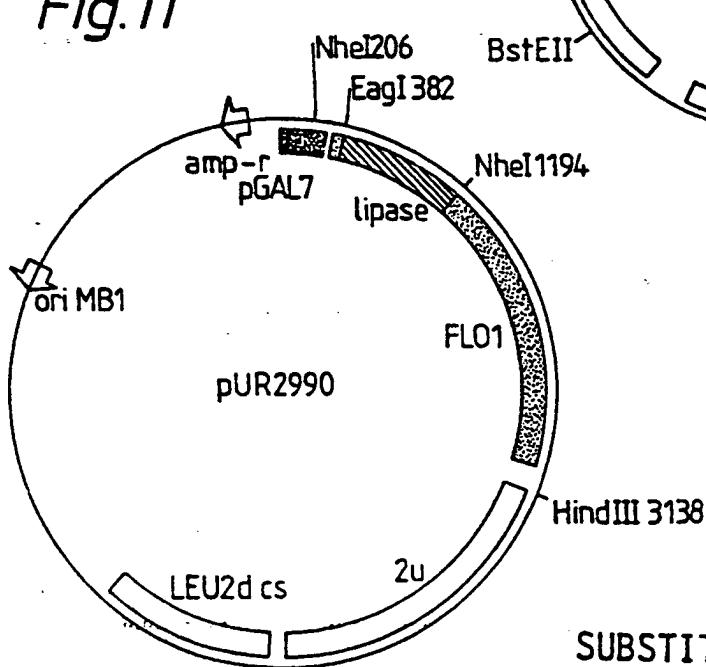


Fig. 12

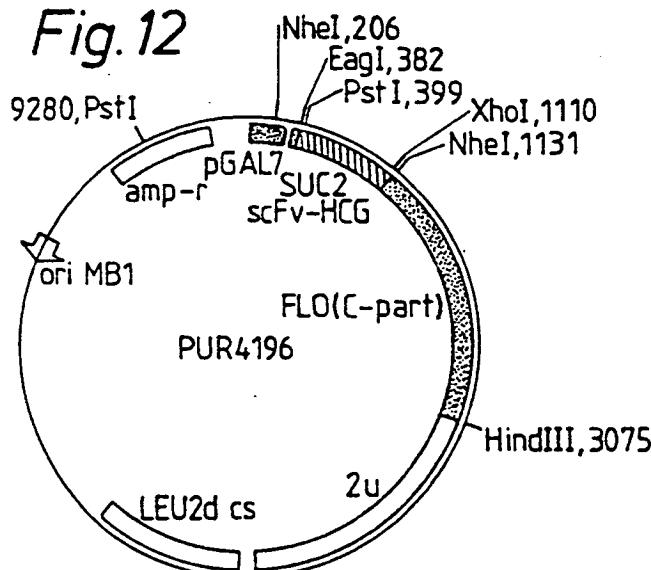


Fig. 13

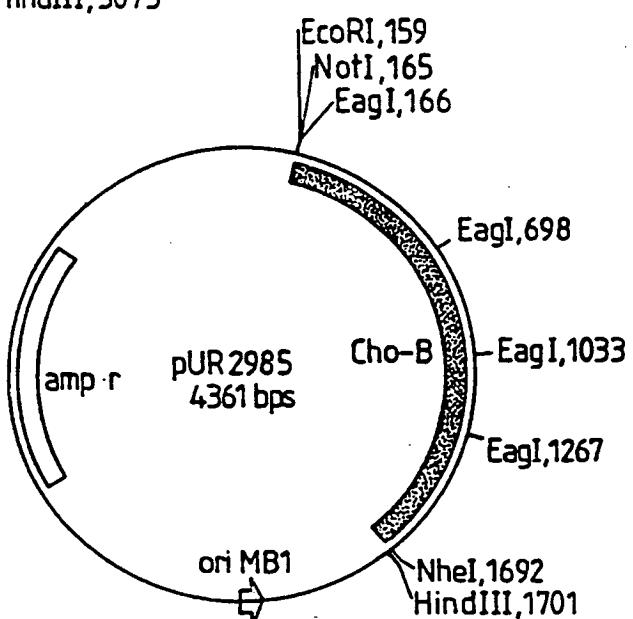
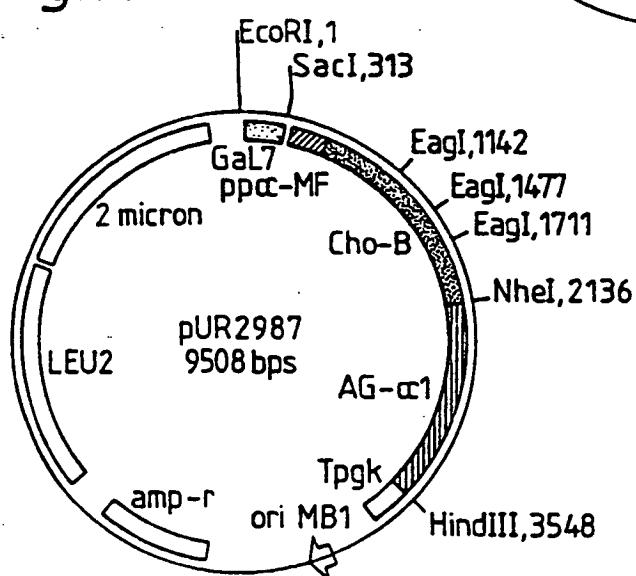


Fig. 14



10/13

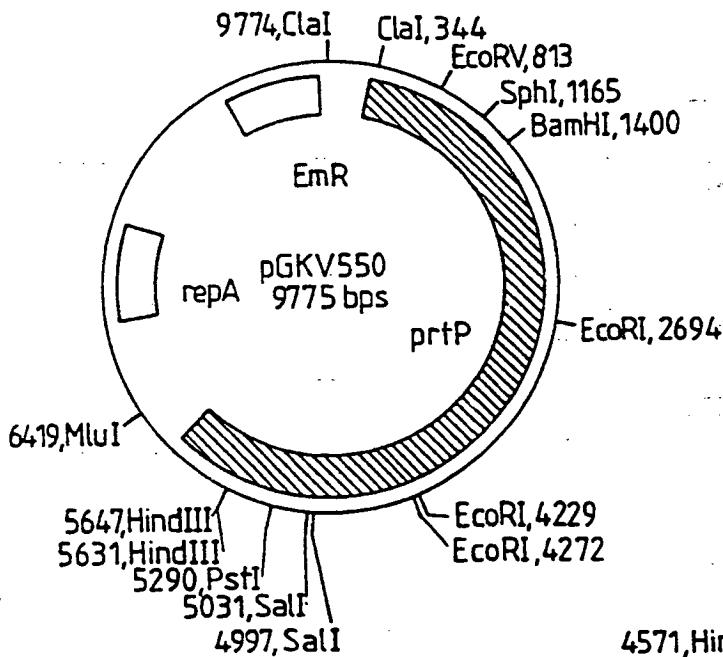


Fig. 15

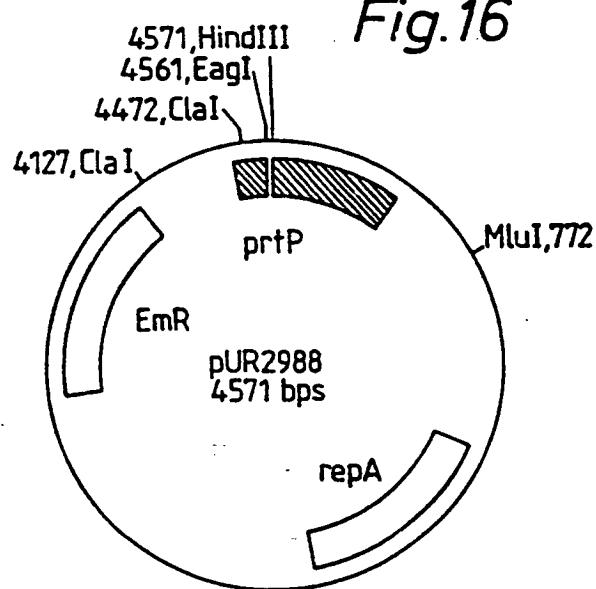
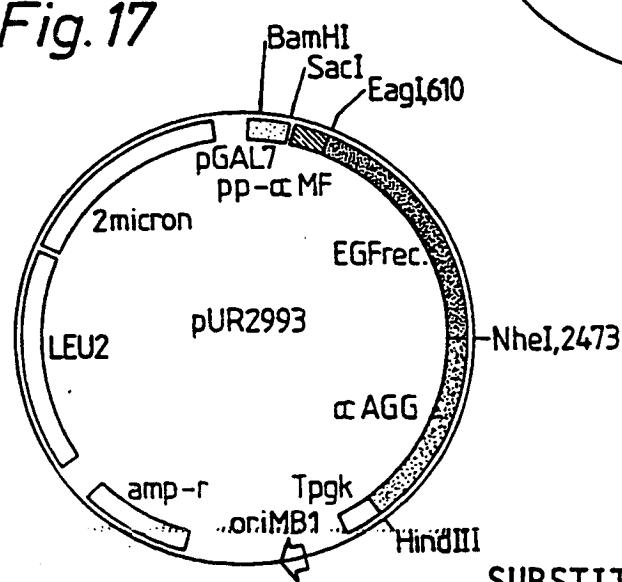


Fig. 16



SUBSTITUTE SHEET (RULE 26)

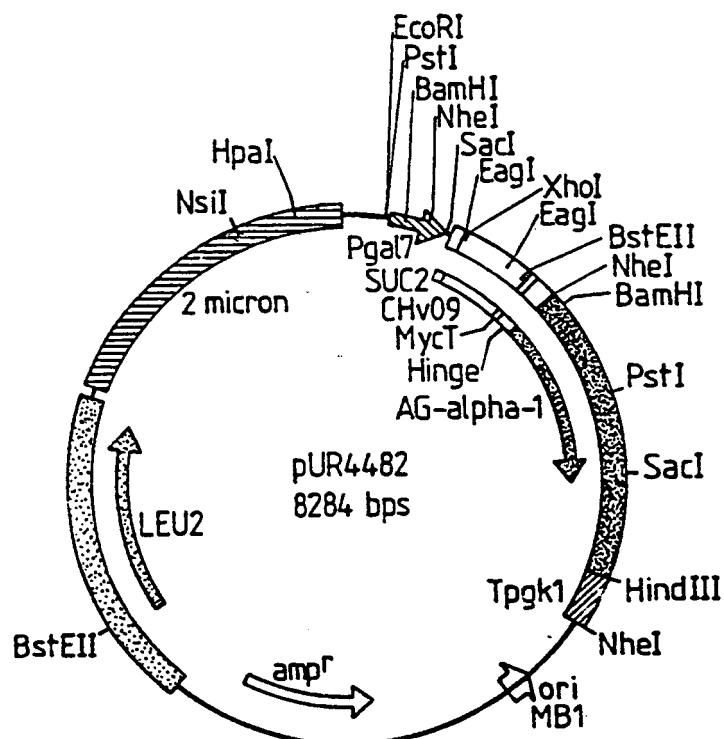


Fig. 18

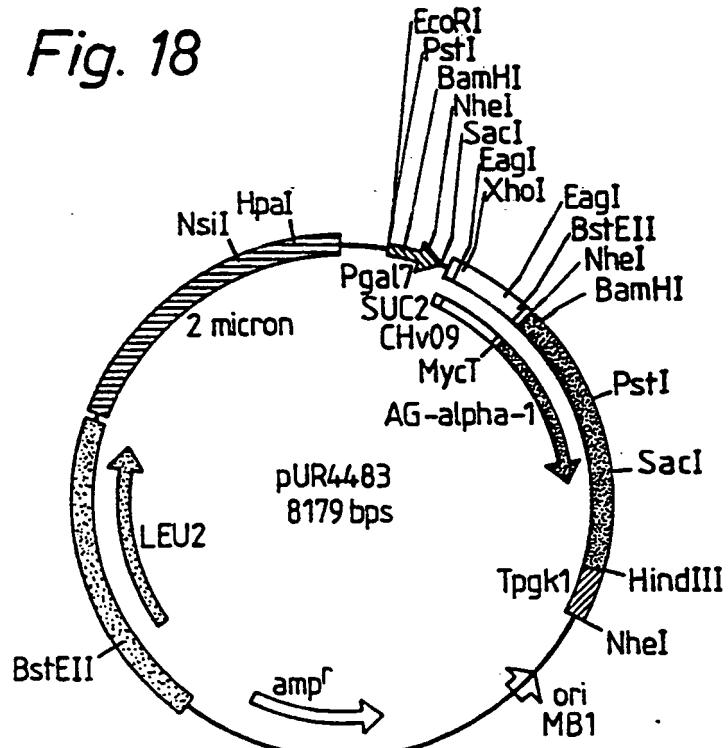
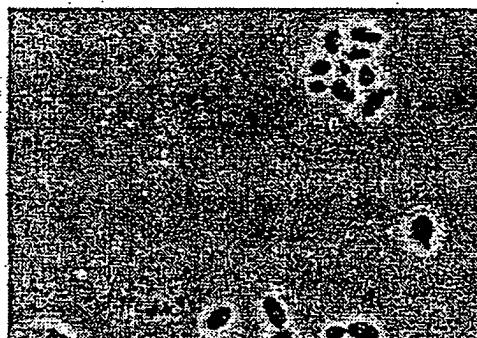


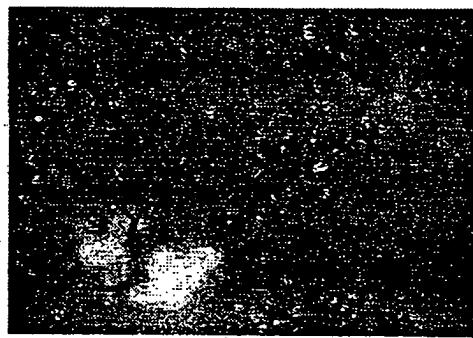
Figure 19



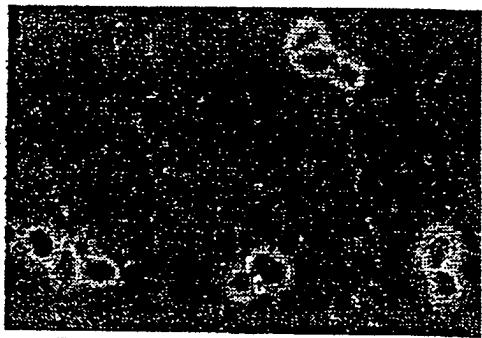
Ph + Fl



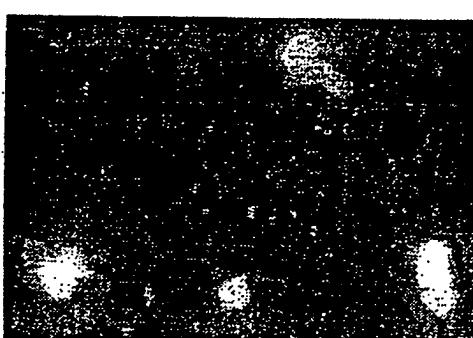
Ph + Fl



Fl

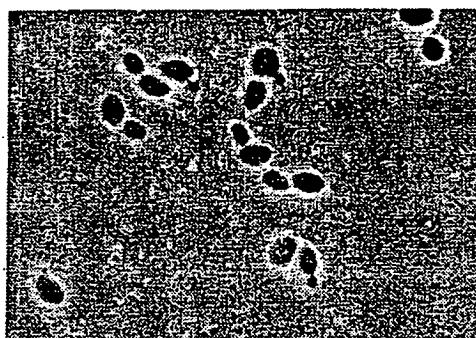


Ph + Fl



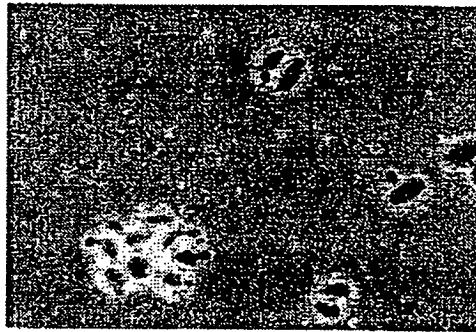
Fl

Figure 20



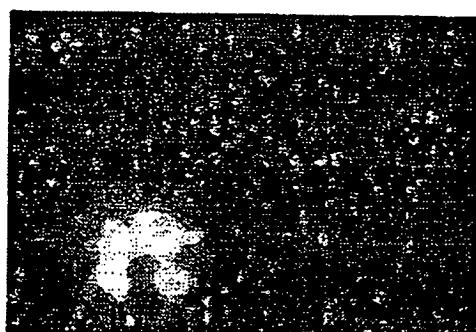
Ph + Fl

pUR4424

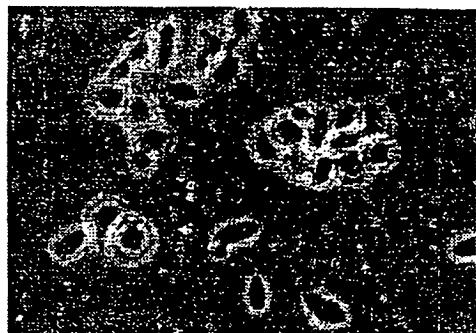


Ph + Fl

pUR4482



Fl



Ph + Fl

pUR4483



Fl

## INTERNATIONAL SEARCH REPORT

Intern: Application No  
PCT/EP 94/00427

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 5 C12N15/62 C12N1/19 C12N1/20 C12N11/16 //C12N1:19,  
 C12R1:465

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 244 221 (GENENTECH, INC;US) 4 November 1987 see the whole document	1,2,4,5, 7,9-16 8
X	WO,A,92 20805 (PIERRE FABRE MEDICAMENT) 26 November 1992 see the whole document	1,2,5,7, 9-14,16 8
Y	WO,A,92 04363 (THE SALK INSTITUTE FOR BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.;US) 19 March 1992 See the abstract	8
P,X	WO,A,94 01567 (UNILEVER PLC,GB) 20 January 1994 cited in the application see the whole document	1-16
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*B\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*A\* document member of the same patent family

1

Date of the actual completion of the international search

Date of mailing of the international search report

25 May 1994

14. 06. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentstaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax (+31-70) 340-3016

Authorized officer

Nauche, S

## INTERNATIONAL SEARCH REPORT

Internat'l Application No  
PCT/EP 94/00427

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US,A,5 223 408 (GOEDDEL, D.V. ETAL.) 29 June 1993 see the whole document -----	1,2,4,5, 7,9-16

1

## INTERNATIONAL SEARCH REPORT

Intern	al Application No
PCT/EP 94/00427	

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0244221	04-11-87	US-A-	4859609	22-08-89
		CA-A-	1328419	12-04-94
		JP-A-	62272990	27-11-87
		US-A-	5030576	09-07-91
WO-A-9220805	26-11-92	AU-A-	1789992	30-12-92
		CA-A-	2103021	01-12-92
		EP-A-	0584167	02-03-94
WO-A-9204363	19-03-92	CA-A-	2090969	05-03-92
		EP-A-	0548267	30-06-93
		JP-T-	6500470	20-01-94
WO-A-9401567	20-01-94	AU-B-	4565393	31-01-94
US-A-5223408	29-06-93	NONE		

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**